IDENTIFICATION OF NOVEL COMPONENTS IN THE DNA DAMAGE RESPONSE PATHWAYS OF *CAENORHABDITIS ELEGANS*

DISSERTATION

ZUR

ERLANGUNG DER NATURWISSENSCHAFTLICHEN DOKTORWÜRDE
(DR. SC. NAT.)

VORGELEGT DER

MATHEMATISCH-NATURWISSENSCHAFTLICHEN FAKULTÄT

DER

UNIVERSITÄT ZÜRICH

VON

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ZÜRICH 2009

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Acknowledgements

I would like to thank Michael Hengartner for giving me the opportunity to do my PhD in his lab. When I met Michael for the first time, he wore a T-shirt with a picture of the invariant lineage of *C. elegans*. The enthusiasm he showed for the worm has inspired me ever since.

Furthermore, I am grateful to my PhD committee Monica Gotta, Joe Jiricny, and Shawn Ahmed for helpful suggestions during the annual meetings. What I appreciated most was that whenever I had difficulties during these last years, I could always call or write and they were always willing to reply with good pieces of advice.

I would like to express my gratitude to the members of the Hengartner lab, for suggestions and for cheering me up when experiments failed, which is not a rare event I have to say. Special thanks to Lilli Stergiou, who introduced me to working with *C. elegans*, to Sabine Schrimpf, who read parts of this thesis, and to Manuel Weiss, who convinced the computer to do what I wanted. I would also like to thank Denise Hengartner for support with bureaucracy.

Many thanks to Alex Hajnal and his lab members for help with time lapse microscopy. I would like to thank Andreas Brachner, Roland Foisner and Yosef Gruenbaum for helpful discussions, encouraging comments and exchange of material.

I am grateful to the Molecular Life Science PhD program and its coordinator Susanna Bachmann. Additionally, I feel gifted to have met my PhD class fellows and hope that we will still be able to keep in touch in the future although we are going to pursue different careers all over the world.

When you pursue a PhD, it does not only require your own dedication, patience, and persistence but also the understanding from the people around you. I would like to express my gratitude to my friends. I am especially indebted to Katharina Hinsch, who has always been there for me when I needed her. I am deeply grateful to my family especially my parents for their continuous support and encouragement.

I am very grateful to Alex for accepting me the way I am, for believing in me, for coping with all the ups and downs during the last months and for so many more things.

For Alex

Summary

Highly sophisticated DNA damage response pathways have evolved to ensure the maintenance of genomic stability. Errors in those pathways have dire consequences for the cell and lead to the accumulation of mutations in the genome, which is a hallmark of tumour cells. Thus, it is of high biological as well as medical interest to better understand the underlying principles and components of these pathways.

In this study, I used the small nematode *Caenorhabditis elegans* as a model organism to identify novel components of the DNA damage response pathways. In a forward genetic screen, I isolated seventeen mutants that are hypersensitive to X-radiation. The strongest defect was observed in *op444*, which I characterized further. *op444* displays F1 embryonic lethality upon ionizing irradiation and UV-C treatment. In response to DNA damage, *op444* initiate cell cycle arrest, apoptosis, and double-strand break repair. Embryos, however, die upon irradiation with defects in chromatin integrity, such as anaphase-bridged chromatin and extranuclear DNA fragments. I found that *op444* maps to *lem-3* (LAP2-emerin-MAN1 domain protein 3), which has not been previously characterized. By expression of a wild-type copy of the gene, I could fully rescue the radiation-sensitvity of *lem-3(op444)*. I also found that *rad-1(mn155)*, whose molecular identity has been unknown so far, is allelic to *lem-3(op444)*. LEM-3 belongs to the GIY-YIG superfamily of nucleases, which have been implicated in recombination and repair.

This work provides the first evidence that LEM-3 acts in processes required to maintain genomic integrity. According to its domain structure, LEM-3 is a putative endonuclease and may be involved in a repair pathway specifically

required in the *C. elegans* embryo. As LEM-3 has been highly conserved throughout evolution, it might also be important to ensure genetic stability in higher organisms.

Zusammenfassung

Eine präzise Weitergabe der genetischen Information ist evolutionär von großer Bedeutung. Zellen verfügen über komplexe Mechanismen, die der Weitergabe von Mutationen entgegenwirken. Sind diese Sicherheitsmechanismen jedoch gestört, akkumuliert die Zelle Mutationen in ihrem Genom und kann sich sogar zu einer Tumorzelle entwickeln. Diese für den Organismus fatalen Auswirkungen zeigen, dass die Erforschung dieser Mechanismen sowohl aus biologischer als auch aus medizinischer Sicht von großer Relevanz ist.

Der Nematode *Caenorhabditis elegans* eignet sich besonders gut für große genetische Studien. In einer solchen habe ich nach Mutanten gesucht, die Fehler in diesen Sicherheitsmechanismen aufweisen. Dadurch sind ihre Nachkommen nicht mehr vor den Schäden durch Bestrahlung geschützt und sterben. Ich habe insgesamt siebzehn Mutanten isoliert, deren Nachkommen nach DNA Schädigung durch Röntgenstrahlen eine eingeschränkte Überlebensfähigkeit aufweisen. Die Mutante *op444* reagiert besonders sensitiv auf Röntgenstrahlung und auf UV-C induzierte Schäden. Einige dieser Sicherheitsmechanismen sind in *op444* immer noch funktionell, im Embryo hingegen entstehen während der Zellteilung defekte Chromatinstrukturen und DNA Fragmente. Durch weitere Experimente habe ich herausgefunden, dass dieser Defekt durch eine Mutation in *lem-3* (LAP2-emerin-MAN1 domain protein 3) bewirkt wird. Durch die Expression einer intakten Kopie des Gens konnte ich den Defekt reversieren. Ich habe außerdem gefunden, dass die vor langer Zeit isolierte, ähnliche Mutante *rad-1* auch eine Mutation in *lem-3* trägt. LEM-3 gehört zu einer Gruppe von Proteinen, die man GIY-YIG Nukleasen nennt. Diese Enzyme spielen eine wichtige Rolle während der DNA Rekombination

und Reparatur.

In dieser Arbeit habe ich gezeigt, dass LEM-3 in *C. elegans* eine wichtige Rolle spielt, um die genetische Information auch nach Bestrahlung noch korrekt weiterzugeben. Meiner Hypothese nach ist LEM-3 eine Endonuklease, deren Aufgabe es ist, spezielle abnormale Strukturen aus der DNA zu entfernen. Dadurch schützt LEM-3 die DNA vor Mutationen. Da die LEM-3 Sequenz evolutionär konserviert ist, wäre es möglich, dass LEM-3 auch in höheren Organismen eine ähnlich wichtige Rolle spielt.

Contents

Part I

Introduction

Chapter 1

Overview

IN THE 1960s, Sydney Brenner realised that for investigating the structure of the nervous system, a new model organism was required. His choice settled on the small nematode *Caenorhabditis elegans* (Brenner, 1974). Thus, he laid the foundation for the success of *C. elegans* as a model system for developmental and molecular biology. Ever since, the science community has appreciated the worm for being "Nature's gift to science", as Sydney Brenner honoured the worm in his Noble lecture in 2002 (Brenner, 2003).

1.1 *Caenorhabditis elegans* as a model organism

C. elegans is a small free-living nematode. It combines a multitude of advantages for the use as a model organism: It has a relatively short life cycle of three days at room temperature. The worm lives as a self-fertilizing hermaphrodite, thus, mutations can be easily maintained in a homozygous state. Furthermore, every cell can be viewed using Differential Interference

Contrast microscopy (DIC), also referred to as Nomarski optics, because the cuticle of the worm and the egg shell are transparent. This led to the discovery of the invariance of the worm's cell lineage, which by now has been well characterised. The existence of excellent forward and reverse genetic tools combined with a sequenced genome make *C. elegans* an invaluable model organism for scientific studies of a plethora of biological processes. One of those processes extensively studied in the worm is apoptosis. A few pioneer studies in *C. elegans* established that apoptosis is a genetically controlled pathway and identified the core protein machinery required (Ellis and Horvitz, 1986; Metzstein et al., 1998; Horvitz and Sternberg, 1982; Horvitz, 1999; Liu and Hengartner, 1999). The fact that those components are conserved even up to mammals additionally underscores the importance of *C. elegans* as a model organism and the impact it has had on biology as a scientific discipline.

1.2 The DNA damage response pathways

When a cell divides, it has to make sure that its daughter cells receive the exact copy of its genome. In other words, it is of utmost importance to preserve genomic integrity during cell division. Extrinsic factors, however, such as ionizing irradiation (IR), UV-C light, or genotoxic chemicals threaten DNA integrity. But even avoidance of such factors does not guarantee genomic stability for the life of a cell. Reactive oxygen species constitute an enemy from within as they arise under normal conditions from the cell's metabolism and alter DNA (Hoeijmakers, 2001).

Consequences of such alterations in the DNA can be of different impact for

the cell or the whole organism. One of the best scenarios is probably that mutations arise in non-coding DNA or, when they are within genes, that they are "silent mutations" and do not lead to any amino acid change on the protein level. In other cases, mutations affect genes and interfere with their functions or obstruct replication or transcription. Those mutations have the potential to lead to a dysfunction or malfunction of cells and their daughters in the context. Cells might accumulate then more and more of such mutations and lose their genomic stability, which is a hallmark of tumour cells. Given those dire consequences DNA alterations can have, it becomes clear that evolution had to come up with a solution to preserve genomic integrity. Our cells intrinsically have elaborate DNA damage response pathways that are activated upon a genotoxic insult. A signal transduction cascade involves proteins that are specialized in sensing damaged DNA (sensor proteins), transmitting this signal, and amplifying it (transducers). In a final step, effector proteins trigger the subsequent response by which the cell stops its cycle, induces transcription, activates DNA repair or even triggers apoptosis (Zhou and Elledge, 2000; Hoeijmakers, 2001; Nyberg et al., 2002; Stergiou and Hengartner, 2004). In this context, the term "checkpoint" is often used. Originally, a checkpoint should be a specific time in the cell cycle where the occurrence of important events (e.g. growth, replication or alignment of chromosomes) is monitored. In some publications, DNA damage checkpoints were defined as pathways that delay or arrest the cell cycle (Sancar et al., 2004). The checkpoint pathway is an internal surveillance system of a cell's well-being. It monitors whether all requirements for the next phase of the cell cycle are fulfilled. When the checkpoint conditions are to the full satisfaction of the cell, the cell cycle progresses to the next phase. The cell is not able to go back in

the cell cycle; thus, the checkpoint control system serves an important function. In case of DNA damage, the checkpoint pathway initiates cell cycle arrest, recruits DNA repair proteins, activates transcriptional responses, and also initiates apoptosis. The checkpoint pathway can be viewed as a regulatory network that elicits appropriate responses upon occurrence of damaged DNA (Zhou and Elledge, 2000). Thus, the term "checkpoint" or "checkpoint pathway" has experienced an expansion (Zhou and Elledge, 2000; Sancar et al., 2004). In *C. elegans*, both cell cycle arrest and apoptosis depend on conserved checkpoint proteins (Gartner et al., 2000; Stergiou and Hengartner, 2004). Therefore, I will use the term checkpoint in a broader sense and not synonymously with cell cycle arrest.

Most of our knowledge comes from studies in the yeasts *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe,* in mammalian culture cells, and mice. Every system has its advantages and limitations. While large scale screenings can be easily carried out in yeasts, mice might reveal more information about the human DNA damage response pathways because of their closer evolutionary relationship.

In Chapter 2, I am going to explain the general principles of apoptosis, then focus on apoptosis in *C. elegans.* In the next chapter, I am going to briefly explain cell cycle arrest and focus on some important key players. Chapter 4 summarizes important aspects of different repair pathways.

Chapter 2

Apoptosis

DURING metazoan development, not only cell proliferation but also the controlled execution of cell death plays an important role. What first appeared to be pure waste of energy and cells, was found to be a highly sophisticated, genetically determined program (Glucksmann, 1951; Horvitz and Sternberg, 1982; Ellis and Horvitz, 1986).

2.1 General aspects

Two main forms of programmed cell death are observed during metazoan development: apoptosis and cell death with autophagy. Apoptosis ("apo" – away, "ptosis" – fall) is usually accompanied by changes in cell morphology, such as shrinkage of the cell, chromatin condensation and nuclear fragmentation (karyorrhexis), loss of mitochondrial membrane potential, and finally detachment of the cellular matrix. The apoptotic bodies or their remnants are then readily phagocytosed by macrophages or neighbouring cells. Unlike

necrosis, apoptosis is not accompanied by an inflammatory response. Autophagic cell death has not been studied well yet. It is usually observed when groups of cells are removed. In contrast to apoptotic cells, autophagic cells contain autophagosomes that finally fuse with the lysosomes to degrade cellular components (Jacobson et al., 1997; Meier et al., 2000; Baehrecke, 2002).

During development, programmed cell death is important for the formation and the deletion of structures, the controlling of cell numbers and the elimination of defective or abnormal cells (Jacobson et al., 1997; Baehrecke, 2002).

Formation and deletion of structures

During animal development several structures are primarily formed and later removed by programmed cell death because they are not needed. The tissue between the developing digits, for instance, is removed by programmed cell death. Another example is the development of the mammalian gonad. Even though the genetic sex is determined from the beginning, development of the early mammal is sexually neutral. To develop the gonad, first, two sets of ducts are formed, the Müllerian ducts and in parallel the Wolffian ducts. In females, the Müllerian ducts develops into the oviducts, whereas the Wolffian ducts are removed by programmed cell death. In males, this fate happens to the Müllerian ducts, as the Wolffian ducts become the vasa deferentia.

Controlling cell numbers

Surprisingly, the number of cells making an organ or tissue at birth is much smaller than the number of cells that was present earlier in development (Jacobson et al., 1997; Buss et al., 2006). Especially during nervous system de-

velopment, excess cells are generated that have to be removed subsequently by programmed cell death. Neurons as well as glia cells are subject to apoptotic culling during development.

Eliminating harmful cells

Programmed cell death is not only used to remove aberrant cells but also to eliminate damaged or harmful cells. The purpose of this "death by design" is not restricted to development but also of utmost importance during later life of animals and humans. Errors during DNA replication or DNA damage can give rise to mutations. To prevent the passing on of those potentially dangerous alterations in the DNA sequence to daughter cells, the cell can activate its intrinsic apoptotic program. Thus, apoptosis is one of the organism's most valuable cancer-prevention mechanism. But not only damaged cells may harm the organism and have to be removed, the development of functional lymphocytes and therefore the functionality of our immune system depends largely on apoptosis (Opferman, 2008). The majority of lymphocyte precursors fails to develop a functional antigen receptor by rearrangement. Consequently, they have to be culled. Lymphocytes with inadequate receptors, i.e. low or too high avidity to major histocompatibility complex molecules, are as well eliminated from the immune repertoire.

After the description of all these beneficial effects apoptosis has for life, it comes without saying that errors in executing apoptosis or an excess of apoptosis have detrimental effects on the organism. Insufficient culling of harmful cells is associated with disorders such as autoimmunity or tumourigenesis. In contrast, an excess of apoptosis is involved in neurodegenerative diseases.

2.2 Apoptosis in the soma of *C. elegans*

2.2.1 General aspects

One of the first observations that was made in research, was the invariance of the cell linage. Thanks to the work of John Sulston the fate of every born cell in *C. elegans* is known (Sulston and Horvitz, 1977; Sulston et al., 1983). Of the 1090 cells that are born, 131 somatic cells undergo programmed cell death. Not only the patterns of cell divisions but also the patterns of cell deaths are highly reproducible. Those dying cells round up and become visible as refractive discs and can be observed using Nomarski optics. The possibility to observe dying cells under the microscope and the fact that apoptosis is not essential for the viability of the worm led to the discovery of several mutants that are defective in the execution of programmed cell death (Ellis and Horvitz, 1986; Hengartner et al., 1992; Conradt and Horvitz, 1998; Metzstein et al., 1998). There are two waves of somatic cell death: More than a hundred cells die during embryogenesis. In a second wave, some neuronal cells are removed during larval stage L2. Extensive genetic studies revealed that programmed cell death happens in four separate processes: In the beginning, the cell decides to die. This process is followed by the actual “killing phase” when the cell autonomously executes its intrinsic apoptotic program. Subsequently, the dying cell is engulfed by one of its neighbours. In the last step, the cell is degraded and no traces of its existence are left in the end.

2.2.2 The core apoptotic machinery

Several genetic screens were carried out to find mutants with abnormal programmed cell death. This led to the identification of the core apoptotic machinery *ced-3*, *ced-4*, *ced-9* (ced = cell death abnormal) and *egl-1* (egg laying defective). These four genes are highly conserved in other species; this again emphasizes how strongly *C. elegans* has contributed to our current knowledge of apoptosis. Strong loss of function mutants of either *ced-3*, *ced-4* or *egl-1* display survival of cells that are usually doomed to die (Ellis and Horvitz, 1986; Conradt and Horvitz, 1998; Lettre and Hengartner, 2006). Thus, these genes have a pro-apoptotic function as opposed to *ced-9*, which has an anti-apoptotic activity. The gain-of-function mutation of *ced-9* blocks apoptosis while the loss-of-function activates programmed cell death in many cells that usually survive (Hengartner et al., 1992) .

2.2.3 Apoptosis activation on the molecular level

To our current knowledge, the most downstream component of the core apoptotic machinery and the final "executioner" of death is the caspase CED-3. Caspases are a special set of cysteine proteases that cleave substrates after aspartic residues. During normal cellular life, CED-3 is kept in an inactive zymogen state. CED-4 is homologue to mammalian APAF-1 (apoptotic protease activating factor-1). CED-9 shows homology to the human proto-oncogene Bcl-2 (B cell lymphoma 2). Bcl-2 also displays anti-apoptotic activity and can even functionally substitute the function of *ced-9* (Hengartner et al., 1992; Hengartner and Horvitz, 1994). EGL-1 is a small BH-3-only-domain protein (Bcl-2 homology domain 3) and belongs to a pro-apoptotic subset of the Bcl-

2 family. It regulates apoptosis by binding to anti-apoptotic members of the Bcl-2 family such as CED-9.

In normal living cells, CED-4 is tethered to the outer surface of mitochondria via an interaction with CED-9. In cells that are programmed to die, EGL-1 is expressed and binds via its BH-3 domain to CED-9. Thereby, CED-9 is altered in a way that CED-4 gets released. CED-4 then shuttles to the nuclear membrane where it forms an oligomer with the pro-caspase CED-3 and activates it (Hirsh et al., 1976; Kimble and Hirsh, 1979). For an illustration of the pathway see figure 2.1.

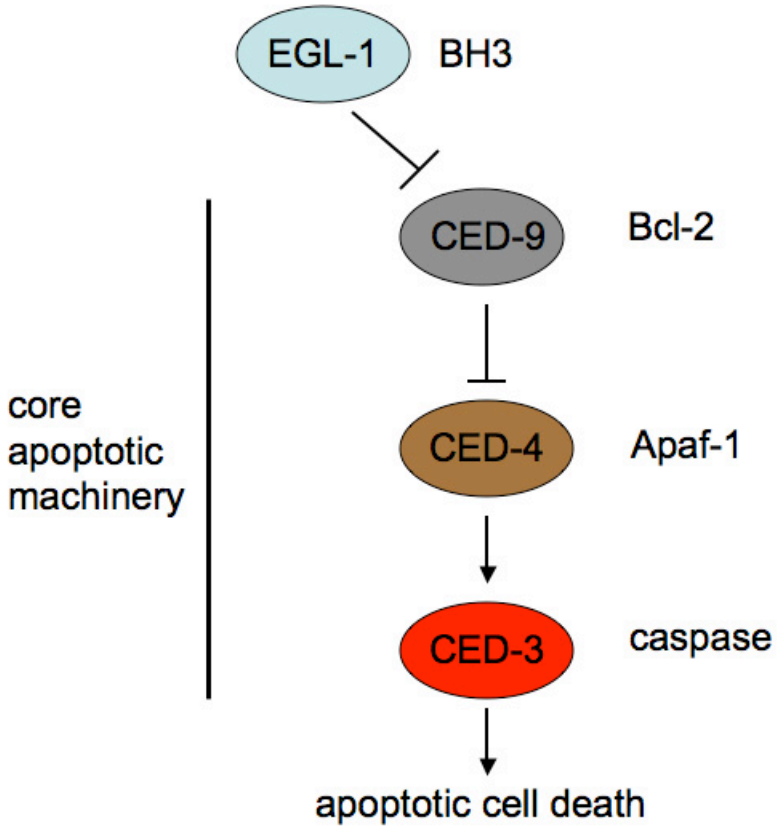


Figure 2.1: **The core apoptotic machinery.** EGL-1 interacts with CED-9 and induces a conformational change. This leads to the release of CED-4 from CED-9 and finally to CED-3 activation.

2.3 Apoptosis in the germ line of *C. elegans*

2.3.1 The germ line of *C. elegans*

The germ line of *C. elegans* is remarkable in many aspects. It is the unique feature of germ cells to pass on their genetic material from generation to generation. Thus, they are often referred to as pluripotent and immortal. During L3 to L4 stage, the hermaphrodites temporarily produce sperm, before they switch to oocyte production. In the following paragraph, I will concentrate on oocyte development as developing sperm cannot undergo apoptosis as far as we know. The gonad of the adult hermaphrodite is symmetrical (Hirsh et al., 1976; Kimble and Hirsh, 1979). It consists of two U-shaped tubular structures – the ovotestes, which are joined together at a common uterus. One of them is shown in Figure 2.2. The distal end of each gonad arm, i.e. the part being distant from the vulva, is capped by a somatic cell – the distal tip cell (DTC) (Kimble and Hirsh, 1979). The DTC secretes the growth factor LAG-2 thereby promoting the mitotic division of the most adjacent nuclei (Kimble and Crittenden, 2007). As they proliferate, they are pushed further down the gonadal tube, until they are finally no longer influenced by the signals from the DTC (Kimble and White, 1981). This leads to a stop of proliferation and entry into meiosis. The transition between mitosis and meiosis is characterised by a crescent-shaped appearance of the chromatin. As the germ cell nuclei travel towards the bend of the gonad, they progress into the pachytene stage of meiosis I. Near the bend of the gonad arm, they exit pachytene, enter diakinesis I and form mature oocytes (Hirsh et al., 1976; Kimble and Crittenden, 2007). Oocytes become fertilized as they are pushed through the sper-

mateca (Hirsh et al., 1976). Meiosis is finally completed after fertilization, and the developing embryo is laid through the vulva. Interestingly, the germ line of *C. elegans* is a large syncytium, in which neither mitotic nor meiotic nuclei are fully enclosed by a plasma membrane; they still maintain a connection to a common cytoplasm via cytoplasmic bridges. What is more, these nuclei develop in a unsynchronized "autonomous" fashion. As these nucleated compartments behave largely as individual cells, the term "germ cell" is commonly used and will also be used in this thesis.

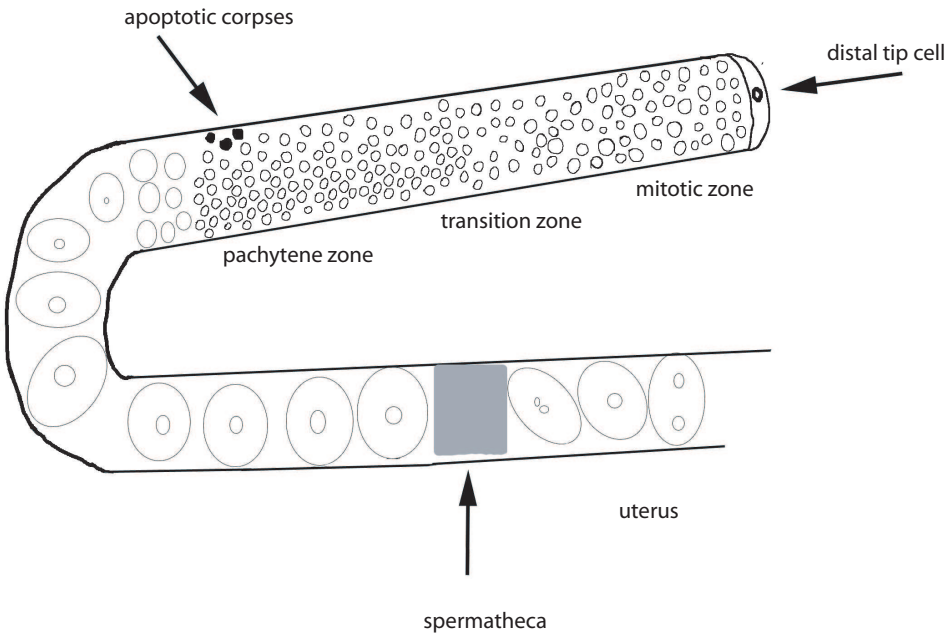


Figure 2.2:

2.3.2 Physiological cell death in the germ line of *C. elegans*

During oocyte development, probably half of all germ cells die of apoptosis (Gumienny et al., 1999). This process is independent of any stress signal from outside and occurs stochastically. Physiological cell death in the germ line

takes place around the bend of the gonadal arm, just before cells enlarge and become oocytes. The dying nuclei become fully enclosed in a plasma membrane. The cytoplasm surrounding them is, however, very small. The apoptotic corpse is then engulfed, not by the neighbouring cell as in the embryo, but by the gonadal sheath cell, which finally degrades the corpse. Molecularly, physiological cell death depends on *ced-3* and *ced-4*. Loss-of-function mutations in *ced-9* cause an increase in apoptosis, however, gain-of-function mutations do not prevent cell death (Gumienny et al., 1999). Another striking difference between physiological germ cell death and developmental cell death is the role of EGL-1. While most, if not all, somatic cell deaths depend on EGL-1, physiological germ cell deaths do not. Exit from pachytene stage of meiosis I requires ras/MAPK signalling (Church et al., 1995). Mutants of the ras/MAPK pathway fail to exit pachytene stage and are defective for physiological cell death (Gumienny et al., 1999). It was shown that the retinoblastoma susceptibility gene orthologue *lin-35* promotes physiological germ cell death by repressing *ced-9* expression (Schertel and Conradt, 2007). It can be speculated that mutations that affect *ced-9* mRNA levels directly or indirectly will show altered levels of physiological cell death. The mechanism, how cells are "chosen" to activate apoptosis and how they actually achieve the activation of CED-3 remains elusive. Why nature culls half of the female germ cells, is still poorly understood. It was shown that the absence of physiological germ cell death negatively affects tissue homeostasis. In strong *ced-3* and *ced-4* loss-of-function mutants where no programmed cell death occurs, the number of germ cells is elevated. They take up the space which is normally devoted to mature oocytes. Thus, the number of oocytes decreases drastically (Gumienny et al., 1999). Whether this is the only reason, is not known. It

is speculated that that those dying cells act as nurse cells for the developing oocytes. The purpose of those cells is simply to synthesize enough cytoplasmic component for oocytes to develop. But how some cells are then "elected" to become oocytes and others are doomed to die, is still not understood.

2.3.3 DNA-damage-induced apoptosis in *C. elegans*

In order to maintain genomic stability, cells damaged too severely are removed. In *C. elegans* we do not see this response in the soma of adult animals. The reason for that is subject to speculation. As adult somatic cells are no longer dividing and, what is even more important, cannot be replaced if they are removed by apoptosis, it would be worse for the organism to remove those cells than to keep damaged cells. This strategy could be called "better malfunction than no function". The germ line, however, passes on cells to the next generation. Accumulating mutations in germ cells would be deleterious for the survival of the species from an evolutionary point of view. By contrast, for fast replicating structures such as viruses, a high mutation rate is a means to evade the immune response and to increase the chances of infection and further replication. For metazoan life, a high mutation rate is usually disadvantageous. In the germ line of *C. elegans,* cells undergo programmed cell death upon genotoxic insults such as ionizing irradiation (Gartner et al., 2000), UV-C light (Stergiou et al., 2007) and DNA damaging chemicals. Apoptosis triggered by DNA damage requires the core apoptotic machinery *ced-3* and *ced-4*. In contrast to physiological germ cell death, no apoptosis is observed in *ced-9* gain of function mutants and the loss of *egl-1* causes a reduction of the apoptotic response (Gartner et al., 2000). Addi-

tionally, another Bcl-2 family member, *ced-13*, is suggested to play a modest role in DNA-damage-induced apoptosis (Schumacher et al., 2005). For an illustration of the pathway see figure 2.3

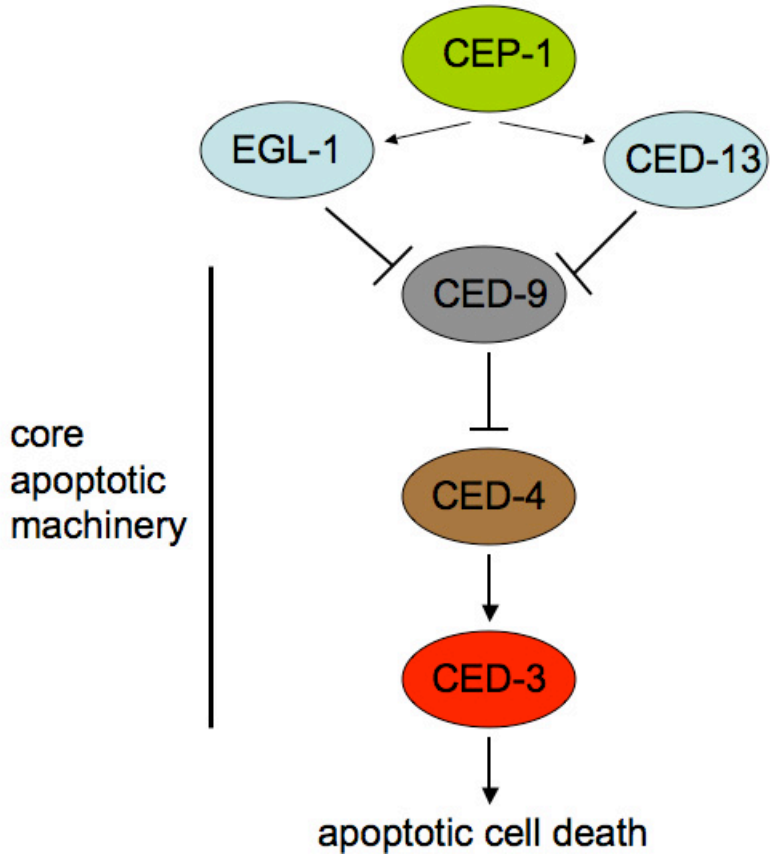


Figure 2.3: **DNA-damage induced apoptosis in *C. elegans*.** Upon DNA damage, CEP-1 induces transcription of *egl-1* and *ced-13*. EGL-1 and CED-13 interact with CED-9, which leads to the release of CED-4 from CED-9 and consequently to an activation of the caspase CED-3.

Several genes have been identified that play a role in DNA-damage-induced apoptosis. In the following paragraphs, I am going to focus on a selection of those that cause a defect only in DNA-damage-induced cell death. A selection of other mutants that also affect cell cycle arrest are discussed later on.

***cep-1*/p53** The tumour suppressor gene p53 has been found to be mutated in more than 50% of all cancers. Patients with Li-Fraumeni syndrome, who have inherited a defect in p53, show predisposition to cancer development. Among other functions, p53 plays a pivotal role in inhibiting cell cycle progression and inducing apoptosis. p53 can activate apoptosis via its function as a transcription factor. More and more target genes are being discovered, among those are famous members as Apaf-1, the BH-3-only domain proteins PUMA and NOXA and various caspases. But p53 also has transcription-independent functions in apoptosis (Pietsch et al., 2008). Two homologous proteins, p63 and p73, were discovered to play mainly a role in development, however, an isoform of mammalian p63 was recently shown to be essential for DNA-damage-induced oocyte death (Suh et al., 2006; Gartner et al., 2008). Extensive searches for a *C. elegans* p53 orthologue did not reveal any obvious candidate. With sensitive algorithms using the p53 of the squid Loligo forbesi as a query and subsequent alignments, finally identified the *cep-1* (C. *elegans* p53 like-1) as the worm orthologue of p53 (Derry et al., 2001). Another group also found *cep-1* by constructing a multiple alignment based search profile (Schumacher et al., 2001). As its mammalian orthologue, CEP-1 acts as a transcription factor (Schumacher et al., 2001). In a loss-of-function mutant of *cep-1,* DNA-damage-induced apoptosis is completely abrogated, cell cycle arrest, however, is not affected by ionizing irradiation but seems to be defective by UV-C (Stergiou et al., 2007). Upon DNA damage, CEP-1 activates transcription of the two BH-3-only proteins that exist in the worm, *egl-1* and *ced-13* (Schumacher et al., 2005). These findings underscore the conservation of *cep-1*, as the mammalian BH-3-only domain proteins PUMA and NOXA are also subject to transcriptional induction by p53. Interestingly, *cep-1* shows

a higher sequence similarity to p63 than to p53. What is more, p63 is required for cell death in oocytes in mammals (Suh et al., 2006). These results are consistent with the idea that p63 is the ancestor of the p53 family of proteins with *cep-1* being more related to p63.

sir-2.1 *sir-2.1* belongs to the conserved family of sirtuins, a family of NAD+-dependent protein deacetylases highly conserved throughout evolution (Greiss et al., 2008). Upon X-ray treatment, *sir-2.1* mutants fail to accumulate apoptotic corpses even though *egl-1* and *ced-13* transcription are normal, implicating that CEP-1 is transcriptionally active. In a similar way as *cep-1*, also *sir-2.1* mutants show a normal cell cycle arrest response. Neither physiological germ cell death, nor developmental cell death in the soma are abrogated in this mutant. More studies are required to reveal the exact level in the pathway where SIR-2.1 acts and what the exact role of this protein is.

abl-1 c-Abl was originally identified as cellular homolog of the v-Abl, the viral Abelson murine leukaemia virus oncogene. It belongs to the family of non-receptor tyrosine kinases and shows conservation from men to worm. Loss-of-function mutations of the worm ortholog *abl-1* cause higher levels of baseline apoptosis in untreated worms (Deng et al., 2004; Gartner et al., 2008). After treatment with ionizing irradiation apoptosis shows a three-fold increase compared to wild-type worms. This massive increase in cell death depends on *ced-3* and *cep-1* indicating that *abl-1* functions upstream of *cep-1*. Interestingly, inactivation of *abl-1* by the c-Abl inhibitor STI-571 (Gleevec), which is used in human cancer therapy, could mimic the phenotype of the mutant. This underscores the usefulness of *C. elegans* as a model system for

the development of new drugs.

Chapter 3

Cell cycle arrest in *C. elegans*

EVERY transition from one cell cycle state to the next is tightly controlled in a cell. The cell monitors certain parameters before it progresses to the next phase. This health test happens at certain checkpoints during the cell cycle. If the cell is ready, it progresses to the next cell cycle phase. In case of problems, such as a break in the DNA, the cell slows down progression. Cells pause at the G1/S checkpoint to prevent replication of damaged DNA (Sancar et al., 2004). When the cell encounters damaged DNA during S-phase, the replication fork stalls. This checkpoint is called the intra-S checkpoint, even though it is not at a transition point for the cell cycle phase. While the cell pauses, the late firing of origins is prevented. In the presence of damaged DNA, the cells can also stop at the G2/M checkpoint, thereby, preventing the passing on of damaged DNA to their daughter cells.

3.1 Cell cycle arrest in the germline of *C. elegans*

Although the gonad is a syncytium, nuclei in the mitotic zone are dividing asynchronously. They differ in cell cycle stage and size. Upon DNA damage, the mitotic nuclei in the distal gonad arm transiently arrest their cell cycle. Nevertheless, they seem to grow and appear enlarged compared to cycling nuclei. A transgenic line expressing the worm cyclin B homolog tagged with YFP, revealed that this cell cycle arrest goes along with accumulation of cyclin B (Speer et al., manuscript in preparation). Thus, it is assumed that the cells arrest in G2/M. The fact that cells of the mitotic zone also arrest upon treatment with hydroxyurea points towards the presence of another checkpoint in S phase. Hydroxyurea is an inhibitor of ribonucleotide reductase and, thus, depletes the dNTP pool of the cell. Consequently, cells respond to this "stress" by stopping their cell cycle.

3.1.1 *hus-1* and *mrt-2*

mrt-2 (mortal germline) was identified in a screen for germ line immortality mutants (Ahmed and Hodgkin, 2000). After 14 generations, this mutant becomes sterile due to progressive telomere shortening. *mrt-2* encodes the *C. elegans* homolog of the *S. pombe* checkpoint gene Rad1. Consistent with its evolutionary conserved role, also *mrt-2* mutants display a defect in the DNA damage responses, i.e. it displays reduced embryonic survival and defective cell cycle arrest after infliction of double strand breaks (Ahmed and Hodgkin, 2000; Gartner et al., 2000). *S. pombe* Rad1 acts together with Hus1 (hydroxyurea sensitive) and Rad9 (9-1-1) in a PCNA (proliferating nuclear

antigen)-like heterotrimeric complex. The PCNA sliding clamp is loaded on sites of ongoing DNA replication and acts as a platform for proteins involved in the replication process (Parrilla-Castellar et al., 2004). The 9-1-1 complex is loaded onto chromatin upon a genotoxic insult. This response is evolutionary conserved from yeast to human. The identification of the *C. elegans* ortholog of *hus-1* as another checkpoint protein was therefore not much of a surprise (Hofmann et al., 2002). *hus-1* mutants also display embryonic lethality and defects in the cell cycle arrest response upon DNA damage (Gartner et al., 2000; Hofmann et al., 2002). The nuclear localization of HUS-1 is dependent on MRT-2 (Hofmann et al., 2002). Upon DNA damage infliction, HUS-1 forms distinct foci on chromatin, which are likely sites of damaged DNA.

3.1.2 *rad-5*

rad-5(mn159) was found in a forward genetic screen for radiation-sensitive mutants (Hartman and Herman, 1982). It is allelic to *clk-2(qm37)*, a slow-growth mutant (Ahmed et al., 2001) and encodes a homolog of *S. cerevisiae* Tel2, a yeast regulator of telomere length. Its function is probably essential, as worms display embryonic lethality at 25°C. Embryonic cell divisions are delayed at restrictive temperature in an ATL-1 (the ATR homolog of *C. elegans*)/CHK-1 (checkpoint kinase 1) dependent manner. Embryos die with elevated levels of RAD-51 foci resulting either from processed DNA double-strand breaks or stalled replication forks. Both alleles are defective in their response to DNA damage, i.e. cell cycle arrest and apoptosis in the germline (Gartner et al., 2000; Ahmed et al., 2001). Double mutants of *rad-5/clk-2*

with either *hus-1* or *mrt-2* do not show any enhancement of the cell cycle arrest defect or the apoptotic defect. They do display, however, enhanced embryonic lethality upon radiation, indicating that the pathway defective in *rad-5/clk-2* is most likely distinct from the 9-1-1 pathway or that *rad-5/clk-2* mutants fail in an additional response, most likely DNA repair (Ahmed et al., 2001). Treatment with hydroxyurea showed that *rad-5*, unlike *hus-1* or *mrt-2*, is defective in the S-phase checkpoint, as their mitotic nuclei fail to induce cell cycle arrest.

3.2 The early embryonic cell cycles

A very particular feature of the early embryonic cell cycles of several species, including *C. elegans*, is the fast alternation between S and M phase combined with the lack of gap phases. The first division produces two cells unequal in size: the larger anterior blastomere, the AB cell, and the posterior blastomere P1. The second cell division happens asynchronously, i.e. the AB cell divides before the P cell. This timing is highly critical for embryonic development, which is underscored by two findings. First, inactivation of components of the replication machinery results in a delayed entry into mitosis (Encalada et al., 2000) with a more profound delay observed in the P1 cell, whose descendants give rise to the future germ line. This leads to patterning defects and finally embryonic lethality. Second, in embryos depleted for *atl-1/chk-1,* the P1 cell divides prematurely, which does not lead to compromised viability but sterility of the animals (Brauchle et al., 2003). Thus, in early embryos the DNA replication checkpoint is “high-jacked” by developmental cues and serves to prevent premature division of the P1 cell. This is also underscored by the fact

that UV treated wild-type embryos still continue their cell cycle unimpeded when high levels of damaged DNA are present. Furthermore, UV exposed animals with a mutation in *rad-3*, the *C. elegans* XPA homolog, which fail to repair UV caused damage, still display cell cycle progression and division timing indistinguishable from wild type (Holway et al., 2006). However, impaired translesion synthesis, eg. in *polh-1* mutants, impairs progression through the cell cycle after MMS induced DNA damage in early embryos (Holway et al., 2006). In summary, DNA damage in the early embryo unlike in the germ line does not cause a delay in cell cycle progression. Rather than stopping the cell cycle and allow repair of the damaged sites, the embryo uses a translesion synthesis pathway to allow replication of damaged DNA.

Chapter 4

DNA repair in *C. elegans*

THE DNA repair pathways are highly conserved throughout evolution. In *C. elegans,* we find the same repair pathways that are active in humans. Below, I summarize what is known about the specific repair pathways in *C. elegans*. As the list of factors involved is growing fast, this description is far from being complete.

4.1 Homologous recombination repair

Homologous recombination (HR) repair is a high-fidelity form of DNA repair used to reverse the deleterious effects of many different kinds of DNA damage, such as double-strand breaks (DSB) or collapsed replication forks (Heyer et al., 2006). Double-strand breaks occur after treatment with genotoxic chemicals and ionizing irradiation. They are also created during meiotic recombination, thus many factors involved in meiotic recombination also play a role in DNA double-strand repair (O'Neil and Rose, 2006). Most mutants

defective in homologous recombination repair display moderate embryonic lethality without any exogenous infliction of damage and an increased frequency of X0 males due to problems during meiosis, such as loss of chromosomes. Most of our knowledge about homologous recombination comes from studies in yeast. In brief, the nuclease activity of Mre11-Rad50-Xrs2/Nbs-1 processes the DSB, which is required for repair to occur (Heyer et al., 2006). The resulting ssDNA is consequently bound by the ssDNA-binding protein RPA (replication protein). This increases the stability of the DNA filament and helps recruiting further proteins. Like Rad51, a member of the RecA-strand exchange family of proteins. This Rad51 nucleoprotein filament promotes then strand invasion and, together with Rad54, generates a physical connection (D-loop) between the homologous DNA stretches (Heyer et al., 2006). In *C. elegans*, *mre-11* mutants show impaired viability and a Him phenotype (Chin and Villeneuve, 2001; Stergiou and Hengartner, 2004). Upon DNA damage, F1 progeny survival is further reduced, radiation-induced apoptosis is elevated, and chromatin fragmentation is apparent in developing oocytes. RNAi of *rad-50*, *rad-51* and *rad-54* display radiation induced embryonic lethality and increased levels of apoptosis (Hofmann et al., 2002; Boulton et al., 2002; Stergiou and Hengartner, 2004; O'Neil and Rose, 2006). In wild-type mitotic germ cells RPA-1 and RAD-51 show a foci-like staining upon DNA damage, consistent with their evolutionary conserved role in double-strand break repair (Garcia-Muse and Boulton, 2005). Additionally, *C. elegans* orthologs of BRCA1 (breast cancer) and BARD1 and BRCA-2 are involved in homologous recombination, as their RNAi depletion shows a Him phenotype and an increase in apoptosis (Boulton et al., 2004). Also CeBRC-2, a BRCA2-related protein, plays a role in HR, as it regulates RAD-51 and

forms foci at sites of DSB (Martin et al., 2005)

4.2 Nonhomologous end joining

In contrast to HR, nonhomologous end joining (NHEJ) is an error prone way to repair a double-strand break. It joins broken ends together putting up with the potential hazard to add or lose nucleotides at the break point. The core components of the NHEJ pathway are conserved in *C. elegans*. The heterodimer KU70/KU80 protects ends from degradation until they are joined together by the action of LIG-4 (ligase 4). Mutations in components of the NHEJ pathway do not show F1 embryonic lethality after irradiation, suggesting that they are not involved in DNA repair in the germline (Clejan et al., 2006). Irradiation of late stage NHEJ mutant embryos produces a variety of Late Egg Rad phenotypes, such as Pvl (protruding vulva) or Egl (egg laying defective), which are absent in mutants of the HR pathway. In contrast to vertebrate cells, HR and NHEJ act non-redundantly in *C. elegans* (Clejan et al., 2006).

4.3 Mismatch repair

The mismatch repair system, which was first identified in *E. coli* mutator strains, removes mismatches, such as nucleotide substitutions or insertions/deletions, in the newly synthesized DNA strand. It is a replication-associated form of repair and also plays a pivotal role in the *bona fide* replication of simple DNA repeat regions called microsatellite DNA, which poses a special challenge to

the replication machinery. Mutations in mismatch repair genes lead to microsatellite instability, a hallmark of many cancers (Jiricny, 2006). *C. elegans* has orthologs of the human mismatch repair genes MSH2, MSH6, MLH1 and PMS2 (Tijsterman et al., 2002; O'Neil and Rose, 2006). Downregulation of *msh-2* and *msh-6* results in a mutator phenotype and microsatellite instability. Two other MutS family members *msh-4* and *msh-5* do not play a role in repair in *C. elegans* but are important for meiosis.

4.4 Nucleotide excision repair

The nucleotide excision repair (NER) pathway is a quite versatile pathway, as it repairs a wide range of structurally unrelated DNA damages. In *E. coli,* the UvrABC endonuclease complex deals with these types of lesions. In brief, UvrA together with UvrB recognize the damage, UvrA dissociates and leaves behind an UvrB-DNA pre-incision complex. UvrC then cuts first 3' and then 5' from the lesion. UvrC belongs to the superfamily of GIY-YIG endonucleases. The GIY-YIG motif is located in the N-terminus of the protein and responsible for the nuclease activity of UvrC (Truglio et al., 2005). The 3' incision can also be performed by Cho (UvrC homolog), another GIY-YIG family member, which acts in combination with UvrC. In this way, some types of damage seem to be repaired more efficiently (Moolenaar et al., 2002; Houten et al., 2002). The helicase UvrD removes the nucleotides, DNA polymerase I fills the gap and DNA ligase acts in a final step in closing the gap. In eukaryotes, we find remarkable parallels to the system in *E. coli*. The pathway seems to involve, however, more than 25 proteins (Hoeijmakers, 2001). Defects in the XP genes central to the NER pathway are the cause of the heredi-

tary cancer-predisposition syndrom xeroderma pigmentosum. The eukaryotic NER pathway can be divided into two different types: global genome repair and transcription-coupled repair. They differ in the very first steps, as they deal with bulky lesions or RNA polymerase blocking lesions, respectively. In *C. elegans*, orthologs for all XP genes exist (O'Neil and Rose, 2006). *xpa-1*, initially found as *rad-3* in a screen for F1 embryonic lethality upon UV-C exposure (Hartman and Herman, 1982), shows UV-C hypersensitivity of late stage embryos and L1 larvae (Astin et al., 2008).

4.5 Base excision repair

Reactive oxygen species from endogenous or exogenous sources cause base damage or modifications. Glycosylases are important enzymes that flip the damaged base out of the DNA leaving behind an AP (apurinic/apyrimidinic) site. The AP endonuclease APE1 subsequently cuts the DNA at that site. The gap is finally filled by DNA polymerase beta and sealed by the XRCC1-ligase 3 complex. The BER pathway and the TCR pathway have some overlapping functions (Hoeijmakers, 2001). In *C. elegans,* NTH-1 acts as a DNA glycosylase, demonstrated by its ability to restore H_2O_2 resistance in *E. coli* glycosylase mutants (Morinaga et al., 2009). *nth-1* mutant worms, however, show wild-type sensitivity to H_2O_2, which is consistent with the assumption that *C. elegans* has (an) additional yet unidentified glycosylase(s).

Part II

Results

Chapter 5

Forward genetic screen for DNA damage response mutants

PREVIOUS STUDIES revealed that a defect in DNA damage responses results in reduced progeny survival rates after mutagenic treatment (Hartman and Herman, 1982). Quantifying embryonic lethality after irradiation of proliferating germ cell nuclei is probably the most sensitive assay to determine defects in DNA damage responses, as it measures the combined effects of cell cycle arrest, apoptosis and DNA repair (Ahmed et al., 2001). The genetic screen I performed to find new genes involved in DNA damage responses was based on this observation.

To create a pool of mutants, I used the genotoxic drug ethyl methane sulfonate (EMS). Details about the mutagenesis and the screening procedure can be found in the Materials and Methods. In brief, individual worms of the F2 generation of the mutagenised animals were transferred to a backup plate to let lay eggs. They were then transferred to a screening plate and subjected to

irradiation. Positive candidates produced dead eggs upon irradiation but their progeny on the backup plate showed only very little lethality. A schematic representation of the screening procedure is shown in Figure 5.1.

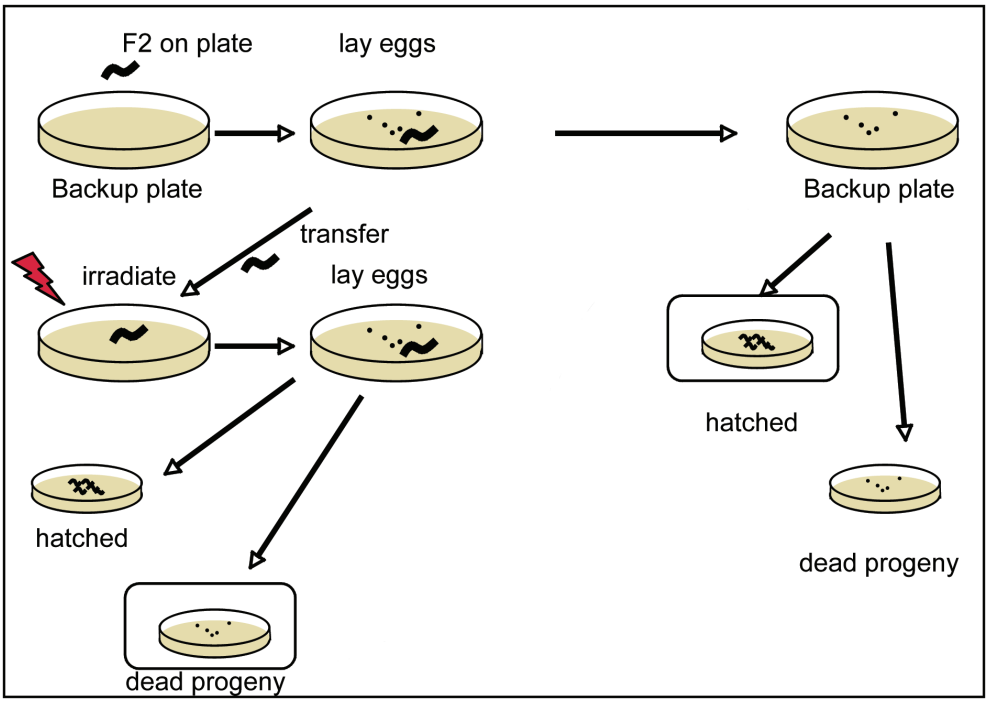


Figure 5.1: **Schematic representation of the screen for radiation-sensitive mutants**. F2 progeny of mutagenised worms were individually put on plates at larval stage four (L4) and allowed to lay eggs for 24 hours (backup plate). They were then transferred to new plates and subjected to X-radiation. Worms were let lay eggs for another 24 hours before they were removed from the plate. Only those worms were kept whose progeny did not survived on the irradiated plate but survived on the backup plate.

In total, 4214 F2 broods were analysed. Of the 148 candidates found positive in the first screening, 17 mutants showed radiation hypersensitivity when reanalysed in triplicate. An initial quantitative examination of the defect in those 17 isolated mutants showed that some of them also displayed embryonic lethality without irradiation (Figure 5.2). This can be explained in several ways. First, embryonic lethality without any genotoxic stress could be due

to inefficient elimination of double-strand breaks that occur naturally during meiotic recombination. This phenotype was observed e.g. when *rad-51* was down-regulated by RNA interference (Takanami et al., 2000; Gartner et al., 2000). Second, the mutants isolated were not backcrossed before this initial quantitative analysis. It is very likely that they have other homozygous or heterozygous mutations in the background, some of which could affect viability in general. Third, we cannot rule out that some of the mutants have a "mutator phenotype", i.e. an increase in the frequency of spontaneous mutations, as it was previously described for *rad-5* (Hartman and Herman, 1982), *msh-2*, *msh-6* and *dog-1* (Cheung et al., 2002). After several generations mutations that could disturb proper function of developmental genes might have occurred.

From these 17 mutants, *op444* showed high F1 embryonic lethality after irradiation and almost no lethality without treatment. There was little variability in this high degree of sensitivity, which was not the case for most of the other mutants. The small standard deviations in the experiment of figure 5.2 emphasize this observation. That is why, I focused on this mutant during my thesis.

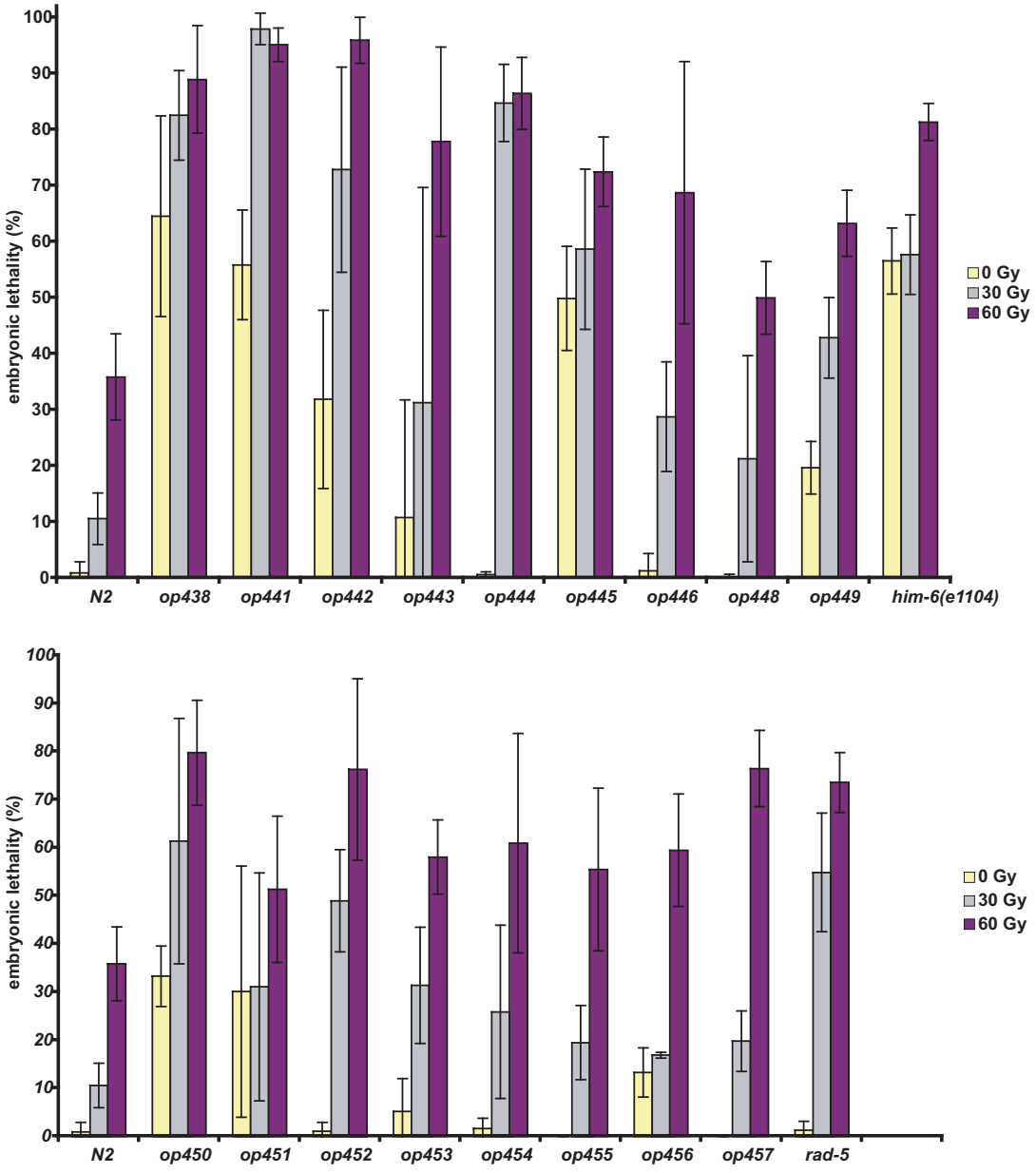


Figure 5.2: **Embryonic lethality of alleles identified in the screen for radiation-sensitive mutants.** Worms were treated with X-radiation 24 hours post larval stage four. Data shown represent the average F1 embryonic lethality of 5 to 8 worms ± S.D.

Chapter 6

Functional characterisation

6.1 F1 embryonic lethality in *op444* is dose dependent

AS THE RADIATION HYPERSENSITIVITY of *op444* persisted to be high after backcrossing the mutant to the Bristol wildtype strain, I decided to focus on this mutant and to start with the initial characterisation. During screening I had found that *op444* was hypersensitive to 60 Gy X-radiation, so it was not clear whether also lower doses of irradiation would cause embryonic lethality. To determine this, I subjected *op444* to 5 Gy, 15 Gy, and 30 Gy, which usually causes very low lethality in the wild type. *op444* turned out to be highly sensitive already at low and very low doses of irradiation (Figure 6.1). The fact that the radiation sensitivity of *op444* increases with the radiation dose further supported the assumption that *op444* is a true DNA damage response mutant.

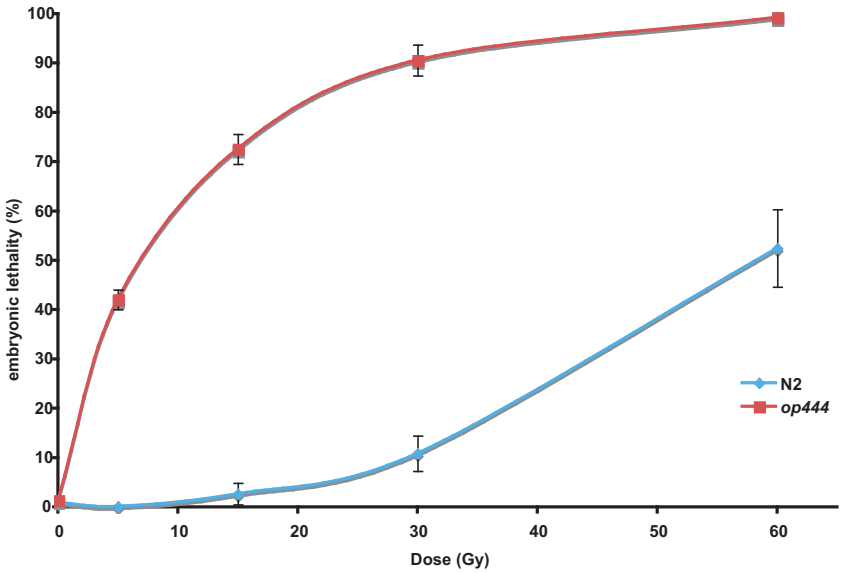


Figure 6.1: **The radiation hypersensitivity of *op444* is dose dependent.** Worms were irradiated with the indicated doses 24 hours post L4 stage, and F1 embryonic lethality was assessed. Each data point represents the average of three independent experiments ± S. D. The progeny of 10 worms were analysed for each experiment.

6.2 The radiation sensitivity of *op444* is stage dependent

In previous studies in our lab embryonic lethality after irradiation was assessed using a different protocol (Hofmann et al., 2002). Worms were irradiated at larval stage four (L4), and embryonic lethality was scored 24 to 36 hours later. To see whether the irradiation time is critical for the percentage of embryonic lethality, I compared both protocols. Interestingly, in contrast to *op444*, the sensitivity of wild-type worms increased when they were irradiated at L4 stage (Figure 6.2). *op444* was found to be hypersensitive to ionising irradiation regardless whether L4s or adults are irradiated, the levels of embryonic lethality, however, differed significantly depending on the

time of irradiation. While the sensitivity of irradiated L4 increased from 20% wild-type level to 51% in *op444*, F1 embryonic lethality of irradiated adults increased from 13% wild-type levels to 92% in *op444*. This result emphasises that the time given for repair and/or the developmental stage at which DNA damage occurs influences the levels of F1 embryonic lethality.

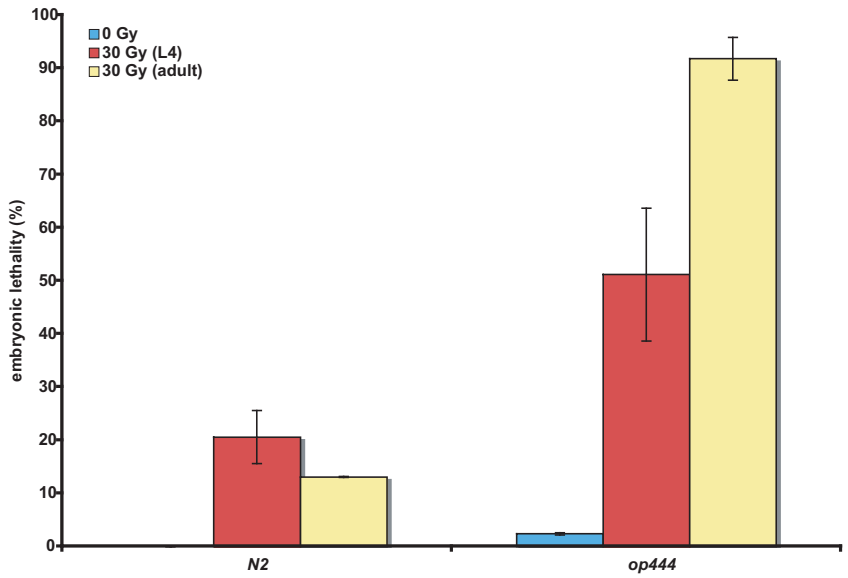


Figure 6.2: ***op444* shows high F1 embryonic lethality when irradiated at larval stage four.** Worms were irradiated with the 30 Gy either at L4 stage or 24 hours post L4, and F1 embryonic lethality was assessed (for details see Materials and Methods). Data represent the average of three independent experiments ± S. D. The progeny of 10 worms were analysed for each experiment.

6.3 *op444* heterozygous animals are not radiation sensitive

Genetic mutations can be dominant or recessive. In a heterozygous state a dominant allele would also give a phenotype, while a recessive allele would behave in a wild-type fashion. The mapping experiments already indicated that *op444* mapped to chromosome I (see CHAPTER 7). The visual marker *dpy-5* is also located on chromosome I and is therefore linked to *op444*. To test the allele for dominance, wild-type males were crossed into *dpy-5(e61) op444*, and the wild-type-looking F1 progeny were analysed for their sensitivity to X-radiation. Exposure of F1 animals to 30 Gy revealed no radiation hypersensitivity of the F1 generation (Figure 6.3 a). Thus, I conclude that *op444* is a recessive allele.

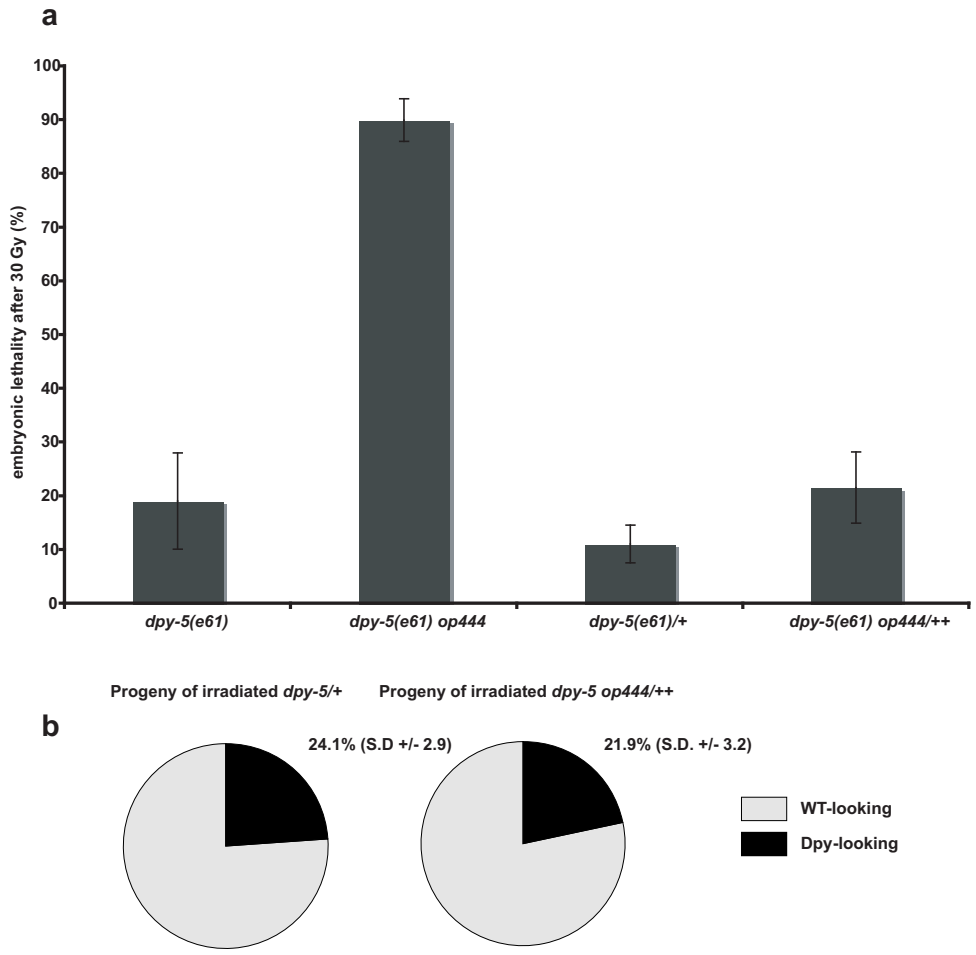


Figure 6.3: **Analysis of animals heterozygous for *op444***. (a) F1 embryonic lethality of *op444* heterozygous animals. Animals were treated with 30 Gy 24 hours post L4 stage. (b) Surviving progeny of heterozygous animals for *dpy-5 op444* or *dpy-5,* respectively. Data represent the average of three individual experiments ± S.D. Progeny of ten worms were scored per experiment.

The F1 lethality of *dpy-5 op444* heterozygotes was still higher than the lethality of F1 embryos from *dpy-5* heterozygotes. This could be the case if *dpy-5 op444* homozygous embryos were more sensitive and would die at a higher rate than heterozygous embryos. To rule out that survival of *dpy-5 op444* embryos was compromised, I analysed the survivors for the presence of the Dpy phenotype, which is linked to the *op444* allele. I found that among the

F1 survivors of irradiated *dpy-5/+* animals 24.1% (S.D. ± 2.9) displayed the Dpy phenotype compared to 21.9% (S.D. ± 3.2) of the survivors of irradiated *dpy-5 op444* (Figure 6.3 b). This result shows that the survival of the F1 *dpy-5 op444* homozygous offspring after irradiation of heterozygous mothers is not compromised and that also the expected quarter of the survivors carries the allele. Furthermore, the survival of *dpy-5 op444* embryos indicates that their radiation-sensitivity is maternally or paternally rescued.

This indicates, first, that homozygous *dpy-5 op444* offsprings are equally susceptible to irradiation as *dpy-5* embryos and, second, that *op444* homozygous embryos are maternally or paternally rescued and survive.

6.4 Apoptosis is normal *op444* animals

Upon irradiation *C. elegans* responds similar to humans, i. e. it induces cell cycle arrest, repair, a change in transcription or apoptosis. The only tissue that is still proliferating during the worm's adulthood is the germ line. In this tissue cell cycle arrest and apoptosis can be easily investigated by using differential interference contrast (DIC) microscopy. One of the cleanest methods to prevent mutations from being passed on to the following generations is the removal of damaged cells by apoptosis. In the meiotic zone of the *C. elegans* germ line approximately half of all female germ cells die even without the external infliction of DNA damage (Gumienny et al., 1999). As observed by DIC microscopy, cells undergoing apoptosis become condensed and increasingly refractive (Gumienny et al., 1999). These structures are termed apoptotic cell "corpses". At any time during the first three to four days of the

hermaphrodite's adulthood two to three apoptotic corpses can be observed. This phenomenon is known as "physiological" germ cell death. Upon DNA damage the number of apoptotic cell corpses highly increases (Gartner et al., 2000). To investigate whether a defect in DNA damage induced apoptosis was the reason for the radiation hypersensitivity in *op444* animals, I irradiated *op444* mutants and measured the extent of apoptotic germ cell death over time. Upon infliction of DNA damage the number of apoptotic corpses increased both in wild type and in the mutant. Without irradiation I observed the usual levels of physiological cell death (Figure 6.4). Therefore, I conclude that *op444* mutants induce a proper apoptotic response upon DNA damage.

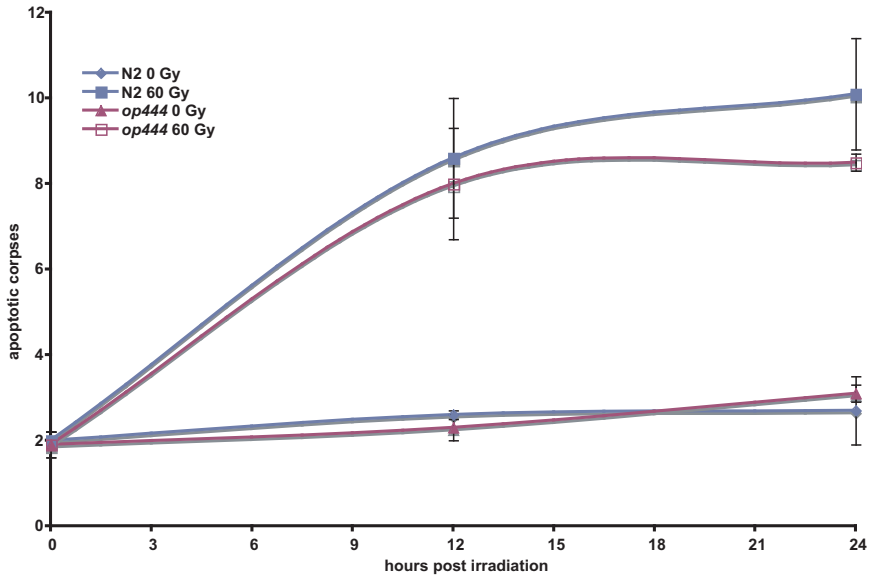


Figure 6.4: **Physiological germ cell death and germ cell death upon irradiation in *op444* animals**. Time course experiment of germ cell apoptosis in wild type and *op444* animals upon irradiation with 60 Gy. Data represent average of three independent experiments ± S. D. (n=10 for each experiment)

6.5 *op444* animals arrest their cell cycle upon DNA damage

Besides apoptosis, exposure to irradiation causes a transient arrest in the cell cycle of mitotic germ cell nuclei. Under unchallenged conditions nuclei in the mitotic zone are in different cell cycle stages; hence, they also differ in their size. Upon exposure to a genotoxic insult the cells cease to proliferate. The size of the nuclei, however, increases. Fewer but enlarged nuclei are found in the mitotic zone of the germ line during cell cycle arrest. Given the importance for a cell to stop cycling when its DNA is damaged, I investigated whether mitotic germ cells of *op444* animals are capable to arrest their cell cycle. I subjected worms to irradiation at L4 stage and measured the diameter of the nuclei in the mitotic zone. Upon DNA damage the average diameter of mitotic nuclei clearly increased in *op444* mutants; hence, I conclude that *op444* mutants have the ability to prevent its mitotic cells from further proliferation after DNA damage (Figure 6.5). In addition, I was interested whether mitotic nuclei start pursuing their normal proliferation program after some time. Twenty-four hours after the insult I observed cells of different sizes in the germ line, which is a clear indication that the nuclei started cycling again (Figure 6.6).

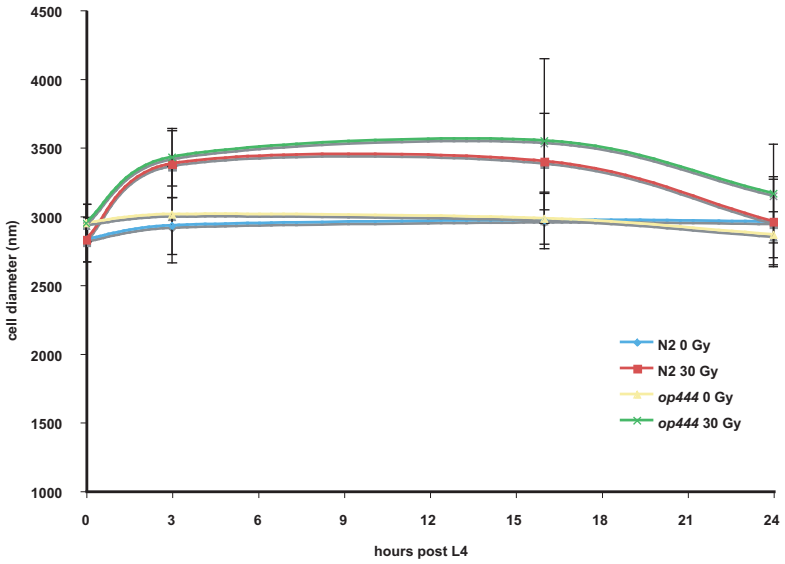


Figure 6.5: **Cell cycle arrest is induced in proliferating mitotic germ cell nuclei upon DNA damage**. Staged L4 worms were irradiated with 30 Gy or left untreated. Data represent average nuclei diameters from 10 to 14 mitotic germ lines ± S. D. Ten nuclei were measured per germ line.

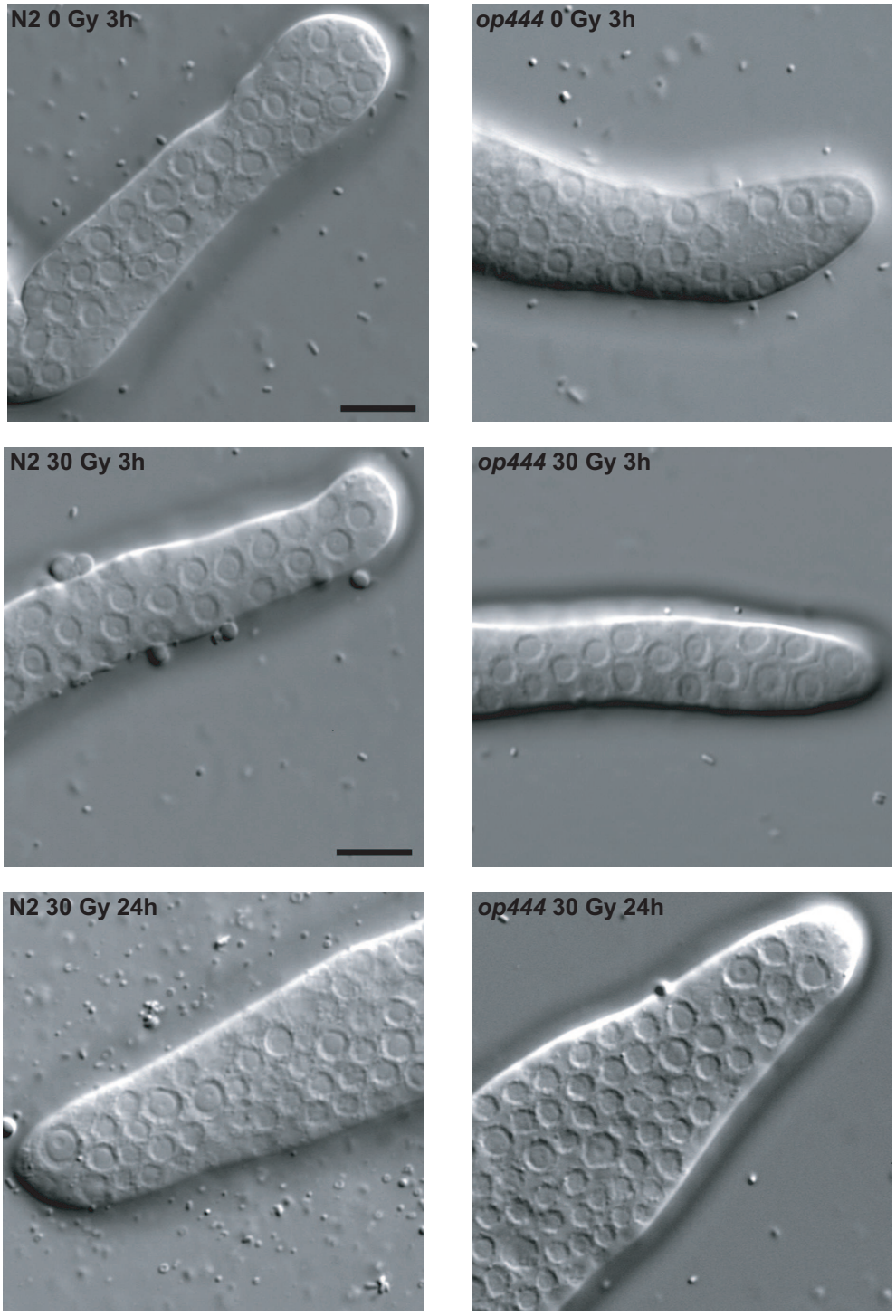


Figure 6.6: **Cell cycle arrest and release in *op444* is normal.** Representative DIC images of wild-type and *op444* dissected germ lines irradiated with 30 Gy at indicated time points or control. Size bar corresponds to 10 μm

6.6 *op444* animals are sensitive to UV-C light

While a nematode is usually not under constant danger of X-radiation exposure, sun-related UV light caused damage poses an environmentally relevant hazard. UV-C light (<280nm) and UV-B light (280-315nm) induce two major classes of genotoxic lesions: cyclobutane-pyrimidine dimers (CPDs) and 6-4 photoproducts (6-4PP), which are mainly repaired by the nucleotide excision repair (NER) pathway. *xpa-1* encodes the *C. elegans* homolog of xeroderma pigmentosum complementation group A and is required in an early step of NER. Mutations in human XPA cause high sensitivity to UV light. In the *ok698* allele of *xpa-1* half of the open reading frame is deleted, and the resulting transcript is degraded, therefore it is likely to be a null allele (Stergiou et al., 2007). *xpa-1(ok698)* and *xpa-1* (RNAi) both cause embryonic lethality upon treatment with UV-C light (Astin et al., 2008; Park et al., 2002). To test whether *op444* also causes sensitivity to damage inflicted by UV-C light, I exposed adult hermaphrodites to 60 J/m^2 and assessed the embryonic lethality of their progeny. *op444* animals were found to be as sensitive to UV-C light as *xpa-1(ok698)* animals (Figure 6.7).

6.7 *op444* appears not to be involved in homologous recombination

To deal with radiation-induced double-strand breaks two main repair pathways have evolved: non homologous end joining (NHEJ) and homologous recombination (HR). It was shown that NHEJ, which is an error-prone form

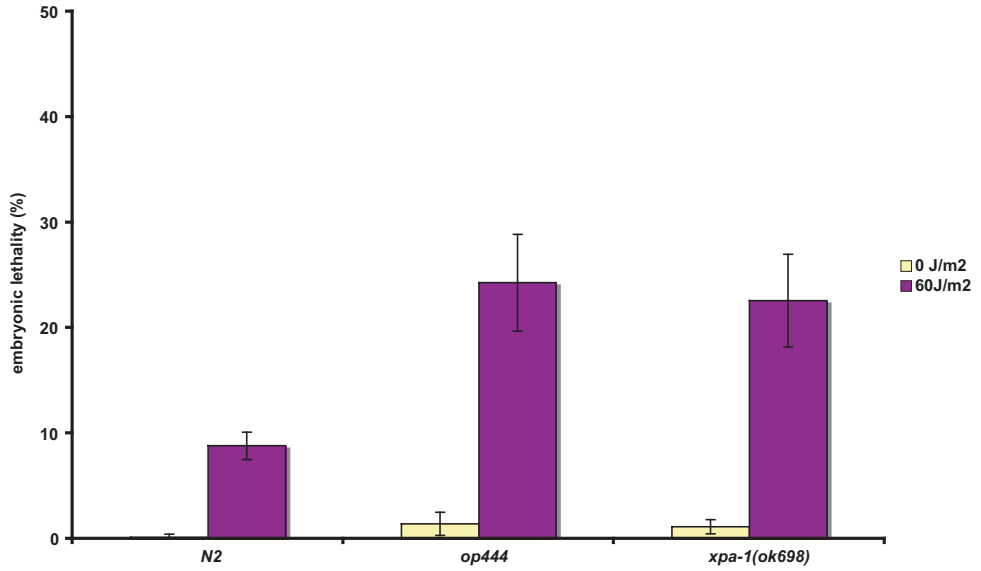


Figure 6.7: ***op444* is sensitive to UV light**. Embryonic lethality of wild type, *op444*, and *xpa-1(ok698)* animals was counted upon 60 J/m^2. Data represent three independent experiments ± S. D. The progeny of 10 worms were analysed for each experiment.

of DNA repair, is the less favourable pathway for the germ line(Clejan et al., 2006), whose task is to faithfully transmit genetic information to the next generation . Thus, homologous recombination is the way of choice to repair DNA damage in the cycling cells of the germ line. Mutants in factors of the HR pathway usually display low levels of embryonic lethality without irradiation and a high incidence of males (Him phenotype), which is caused by XX non-disjunction in hermaphrodites leading to an increased occurrence of X0 males (Hodgkin et al., 1979) Neither a basic level of lethality nor a higher incidence of males was observed in *op444* animals. This observation is a first indication that *op444* does not cause a defect in homologous recombination.

6.8 RAD-54 foci formation in *op444* animals upon irradiation

As I had found that the cell cycle arrest response and the apoptotic response in *op444* animals were wild-type, I wanted to investigate whether repair was induced normally upon irradiation. I took advantage of a transgenic line that expresses RAD-54::YFP (made by Dr. Lilli Stergiou). RAD-54 is highly conserved in eukaryotes (Heyer et al., 2006). It interacts with RAD-51 and plays an important role in homologous recombination and repair. In *C. elegans* RAD-54 localizes in a dot-like pattern to meiotic cells in the germ line. In *op444* animals, RAD-54 foci were found in the meiotic zone of the germ line, indicating that homologous recombination was not affected by the *op444* mutation. This is in line with the observation that there is no Him phenotype in *op444* mutants.

Upon X-radiation treatment of young adults RAD-54 forms distinct foci at sites of active repair (Stergiou et al. manuscript in preparation). These foci can be found in the mitotic as well as in the meiotic zone of the germ line. To determine whether *op444* induced homologous recombination repair in mitotic cells, I inflicted damage with X-rays in the germ line of *op444* and wild type and quantified the number of induced foci. RAD-54 foci formed with the same frequency in *op444* and as in wild-type animals (Figure 6.8). This shows that homologous recombinational repair is successfully induced in *op444* animals.

As not only the initiation of repair but also the successful completion of repair is important for the cells' well-being, I determined whether RAD-54 foci were

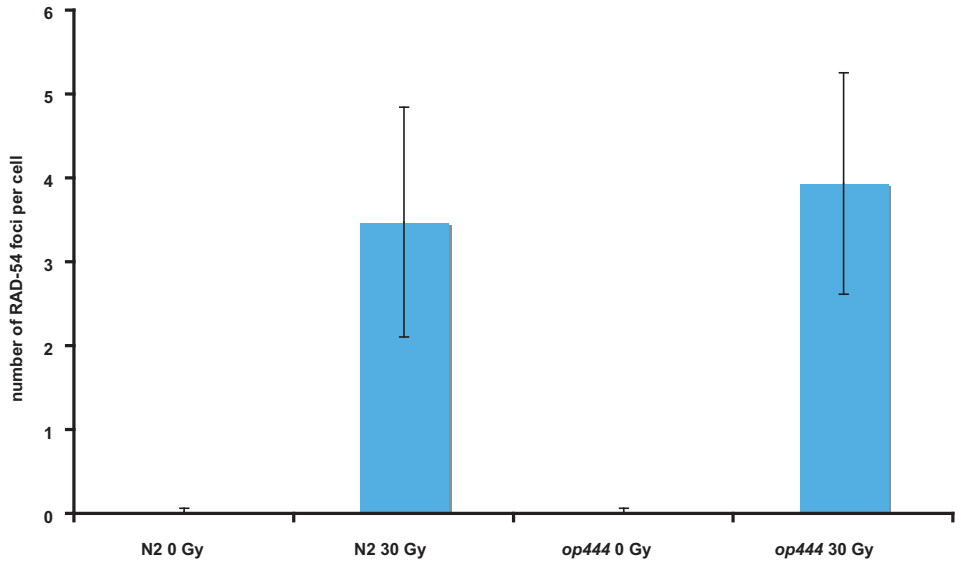


Figure 6.8: **Homologous recombinational repair is induced in *op444* animals**. RAD-54 foci were counted in mitotic germ cell nuclei after treatment of young adult worms with 30 Gy. 8-23 nuclei were counted per germ line. n = number of germlines. n = 14 for N2 (30 Gy) and *op444* (30 Gy), n = 27 for N2 (0 Gy), n = 28 for *op444* (0 Gy).

removed after time was given for repair. Seventeen hours after irradiation, in wild type and in *op444* animals some cells were still positive for RAD-54 foci (Figure 6.9). Interestingly, those cells also had an enlarged appearance, indicative for cell cycle arrest. In many cells, however, RAD-54 foci could not be detected any more. Those cells also looked smaller, which is in support of the assumption that they resumed their normal mitotic proliferation.

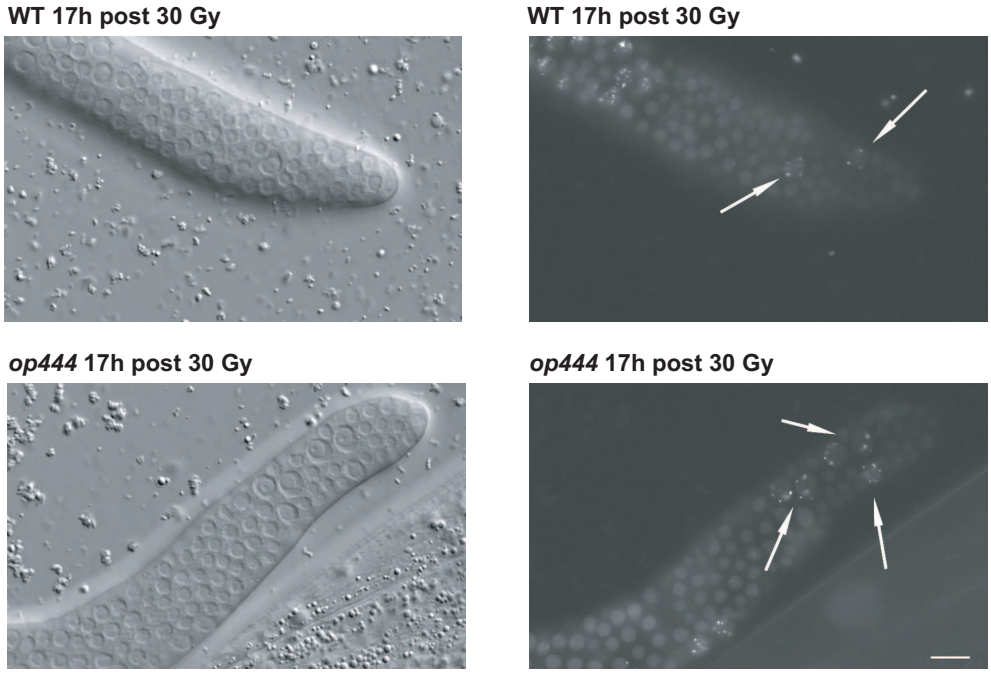


Figure 6.9: **RAD-54 foci are removed from nuclei after time for repair.** Images were taken 17 hours after irradiation with 30 Gy. Size bar corresponds to 10 μm.

Chapter 7

Mapping

A FORWARD GENETIC SCREEN can reveal important aspects of biological pathways. Screening itself is often tedious and requires a lot of time and perseverance. However, when an interesting mutant candidate is found, the goal is only partially achieved. The actual work, however, is then only about to start. To have a true genetic mutant with a phenotype of 100% penetrance from a forward genetic screen is on the one hand a gift on the other hand finding the gene affected in this mutant represents a tremendous challenge to a researcher's endurance. Especially if the phenotype is not readily visible, the time investment into mapping should not be underestimated.

For mapping the mutants had to fulfill the following criteria: First, they should display high embryonic lethality upon irradiation and low or moderate lethality without treatment. This is important to easily follow the phenotype. Second, the development should not be delayed compared to wild type, which is important for maintaining the strain and carrying out comparative experiments. *op444* mutants were first crossed to the wild-type strain in order to get

rid of other mutations that might have been inflicted during mutagenesis. Additional mutations that are closely linked to the mutation of interest, however, are rarely eliminated by this method. To determine linkage to a chromosome, I used two different approaches. At first, I wanted to assign *op444* to a linkage group taking advantage of the existence of fragment length polymorphisms (FLPs) and single nucleotide polymorphisms (SNPs) between the CB4856, a *C. elegans* strain isolated in Hawaii, and the commonly used N2 Bristol wild-type strain (Wicks et al., 2001; Zipperlen et al., 2005). The polymorphic Hawaii strain displayed radiation hypersensitivity similar to the Bristol wild type (Figure 7.1).

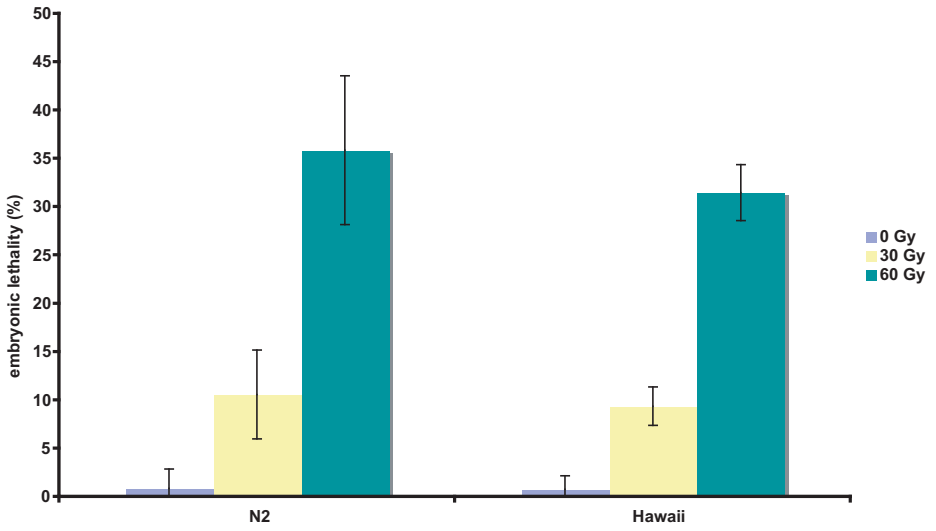


Figure 7.1: **Embryonic lethaltiy for Bristol (N2) and CB4856 (Hawaii).** Data represent average F1 lethality ± S. D. The progeny of 7 to 9 worms were analysed.

For SNP mapping Hawaii males were mated to *op444* animals; the F2 generation was allowed to lay a few eggs, before they were transferred and irradiated. By determining embryonic lethality of the F3 generation, animals were

grouped into *op444* positive and *op444* negative animals. For Tier 1 lysates of *op444* positive F3 animals were analysed for the presence of "Bristol polymorphisms". For this initial analysis one FLP assay per chromosome arm was used. *op444* showed clear linkage to chromosome I (Figure 7.2).

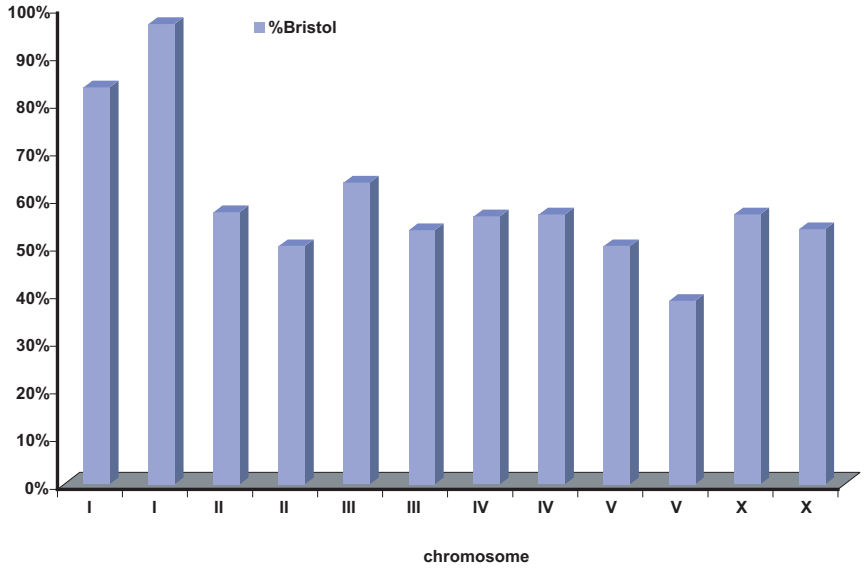


Figure 7.2: **FLP mapping of *op444***. Analysis of the *op444* mutation shows linkage to chromosome I.

In a second approach, I crossed *op444* mutants to two commonly used mapping strains, MT3751 (*dpy-5(e61) I; rol-6(e187) II; unc-32(e189) III*) and MT464 (*unc-5(e53) IV, dpy-11(e224) V; lon-2(e678) X*). Wild-type looking F2 generation were allowed to let lay and were then tested for *op444* by irradiation. The progeny of *op444* homozygous mutants were examined for the presence of one or more visual markers. The under-representation of the *dpy-5* marker in the progeny (Table 7.1) further supported the result of Tier 1 that *op444* is linked to chromosome I.

Table 7.1: **Classical linkage analysis**. Two-factor mapping reveals a linkage of *op444* to linkage group (LG) I.

marker	**LG**	***op444* positive**	***op444* positive segregating marker**	***op444* positive segregating marker (%)**
dpy-5(e61)	I	24	3	12.5
rol-6(e187)	II	24	15	62.5
unc-32(e189)	III	24	15	62.5
unc-5(e53)	IV	8	4	50
dpy-11(e224)	V	8	3	37.5
lon-2(e678)	X	8	6	75

As Tier 1 showed a higher linkage to the right arm of chromosome I, more SNPs in this region were analysed. Tier 2 revealed that *op444* mapped between the marker ZH1-01 (nucleotide position 8416904) and ZH1-23 (nucleotide position 9799636). The two morphological markers *unc-13(e51)* and *dpy-24(s71)* flanked that interval and were used to build a triple mutant. This mutant was then crossed again to the Hawaii isolate. Recombinants between *unc-13(e51) op444 dpy-24(s71)* and CB4856 placed *op444* between the molecular positions 9561849 bp and 9581715 bp. This roughly 20 kb-interval contained six genes: *lem-3, rig-5, C36F7.5, F42H11.1, C36F7.t2, C36F7.t3*. At that time no mutants for these genes were available, hence, I tried to knock them down using RNA interference (RNAi). Feeding of the corresponding RNAi clones did not result in embryonic lethality upon irradiation. As some genes are less susceptible to knockdown by feeding RNAi, I had a closer look at the functions of the genes in the interval. *C36F7.t2* and *C36F7.t3* are t-RNA genes, *C36F7.5* and *F42H11.1* are predicted as homologs of human collagen, *rig-5* is predicted to be a neuronal gene. The gene *lem-3* encodes a protein containing ankyrin repeats, a LEM (LAP2 Emerin

Man1) domain and the protein domain COG3680, which is a domain conserved in bacteria with unknown function. To me, *lem-3* appeared to be the most likely candidate because of the domain composition, thus, I started to sequence the gene. Sequence analysis of *lem-3 (F42H11.2)* in *op444* animals revealed a guanine to thymine transversion at base pair +2363, resulting in a leucine to phenylalanine substitution in the C-terminus of the protein (Figure 7.3).

Figure 7.3: ***op444* maps to *lem-3***. Boxes depict exons, the position of the *op444* mutation is indicated with a vertical line.

Chapter 8

Gene and protein structure

8.1 Gene structure of *lem-3*

The *lem-3* gene, as predicted in the gene model of WormBase Release WS200, is composed of eight exons. The prediction programs Twin Scan and mSplicer, however, do not predict the 84bp long exon four. This exon is not found in *C. briggsae* either. This prompted me to sequence three available expressed sequence tags (ESTs) that were kindly provided by the Kohara lab. The EST clones yk1506 and yk1570 did not contain exon 4. The EST clone yk1637 contained exon 4 but flanked by introns. Most probably this clone corresponds to a splice intermediate. When I amplified the open reading frame of *lem-3* from a cDNA library, exon 4 was missing again. Thus, I conclude that exon 4 is mispredicted in the current Gene Model and that the true model is the one of Twin Scan and mSplicer, which predicts 2115bp for the open reading frame and 7 exons in total (Figure 7.3). What is more, *lem-3* is the last gene of the operon CEOP1548 and has a predicted SL2 splice site. The

two other genes in the operon are *K07A1.1* and *K07A1.17*, neither of them has been characterised so far.

8.2 Conserved protein domains

To learn more about the function of LEM-3, I analysed the protein sequence for the presence of conserved domains with two different programs: Pfam 23 (http://pfam.sanger.ac.uk/) and the Simple Modular Architecture Research Tool SMART (http://smart.embl-heidelberg.de/) (Schultz et al., 1998; Letunic et al., 2006)

Pfam predicted two ankyrin repeats (AA32-62 and AA63-95) and a LEM (domain (AA426-469). SMART found as well two ankyrin repeats (AA28 – 59 and AA63-92) and a LEM (LAP2, emerin, MAN1) domain (AA426 - 470) together with three regions of low complexity. Additionally, SMART predicted different other domains with low or very low E-values. Among those was a GIY-YIG domain (AA526 – 636) with an E-value 4.12e+00 (Figure 8.1).

Ankyrin repeats

Ankyrin repeats are relatively conserved motifs, which mediate protein-protein interactions and are involved in many different biological functions. An ankyrin motif always exhibits a helix-turn-helix conformation (Li et al., 2006). Ankyrin repeats are rather frequent motifs, according to SMART, 98 *C. elegans* proteins share this motif. *C. elegans* LEM-3 has two ankyrin repeats, other orthologs have up to four repeats.

LEM-3mSplicer
705 aa

MPPNGAITTT PRSRMPPTTP SSGKSRPKKE TLHYLAASSS TTSVDAARTL
LERGANVNAI DRDGATPLHY ACTHDNVAMA QLLLTFGADP MSADKLGRTA
YSIAKGNTLR FLRRYKKSSN RQRLGFFRRF FACHSRNETF FIVRNNQEVA
PLRPTALAEA ASISFNRGNV LTNSYRCAKK KIRATFHAIR RSRSNSTATL
QDVVLTSEGI RTVTTPSRRA PKATVYAKRS MSVSDLLLIP DRRDIKNEDV
KTRGSPVKKT RGTGRSRTPE AILNPRKQRT PVNHHKRSKS QETKLVAMPS
PNSMAYYNTS RARNAGLRPA PSAPPLSPEP AIAAVKTPKT PTTPKTSKGR
SKSPANTTAY FTADESLELL GNNMEKLNIE SKSAKSSKLK TPSKPTTSSS
TSFSSAEDDK EAEVSTPTTV DDGEIRKIRR LREGELKSEL KKFGISPAGP
LDARTRRLYE KKLLIERRKI TNRGYSPDAD VVSCRNSPQL ELVLRNGFLP
ADFASRARKC DENVRSEFSG NGFGYNAFCY LIMDPRILGS NVENLTLETF
VRSIFYVGKG SKNRPLAHFI DARNERRDKL DKLKTCEKLK TIDELWTLGF
GIPRHEISHG VSDEEAFVRE ASIIEAVKLK NLRNKKAGEF HGTTKSWDNI
TKSEYGTFLL DRALSTLKLE GIRLITEDNL PDSLYPYVNN RRGAGGGRTP
KTPK

Figure 8.1: **Conserved domains in LEM-3.** LEM-3 (mSplicer predicted) contains three different conserved domains: two ankyrin repeats shown in green (ANK), a LEM domain shown in pink (LEM) and a GIY domain of the subtype COG3680 shown in red (GIY)

LEM domain

The LEM domain is an approximately 40 aa long motif which is found in proteins of the inner nuclear membrane, including LAP2, emerin, and MAN1, hence, its name (Lin et al., 2000). LEM-domain proteins show evolutionary conservation but are absent from yeasts and plants (Wagner and Krohne, 2007). Those LEM-domain proteins that are part of the inner nuclear membrane interact with A-type lamins or B-type lamins. LEM domains of all proteins characterised bind to a highly conserved protein called BAF (barrier to autointegration factor). This 10 kD protein acts as a host component in preintegration complexes of retroviruses. This complex enables the virus to integrate into foreign DNA and blocks autointegration (Lee and Craigie,

1994, 1998). Not only does BAF bind LEM domain proteins, but also ds-DNA in a sequence-independent manner (Zheng, 2000) and lamins. BAF has multifaceted biological roles. It is a key component of viral integration, nuclear assembly, chromatin condensation, and gene expression (Margalit et al., 2007b,a; Wagner and Krohne, 2007). Mutations in lamins and LEM-domain proteins lead to genetic disorders that are collectively called laminopathies.

In *C. elegans* there is only one gene encoding lamin *lmn-1*, a B-type lamin. There are three LEM domain proteins known so far: EMR-1, LEM-2, and LEM-3. EMR-1 and LEM-2 share similar architecture, both have a LEM domain at the N-terminus and one or two transmembrane domains, respectively. LEM-3 shows a different domain composition. Its LEM domain is located in the middle of the protein, and there is no evidence of a transmembrane domain. Downregulation of *baf-1* results in defects in chromosome segregation, anaphase bridges, and abnormal chromatin condensation. Embryos die at around 100-cell stage (Zheng et al., 2000). Lamin, emerin, and LEM-2 fail to assemble properly on the nuclear envelope and instead show a patchy staining pattern. (Margalit et al., 2005). Noteworthy, also downregulation of *lmn-1* leads to similar phenotypes. In those cells EMR-1, LEM-2 and BAF-1 fail to assemble around chromatin (Liu et al., 2000, 2003). Mutations in *emr-1* or *lem-2* do not cause any obvious phenotype, but simultaneous downregulation of both causes the mislocalisation of lamin and BAF-1 and again leads to chromosome segregation defects.

GIY

The GIY-YIG domain was originally found in homing endonucleases. It has two short motifs "GIY" and "YIG" followd by an arginine and a glutamate residue (Dunin-Horkawicz, 2006). Members of the family include endonuclease II of the T4 phage, mobile genetic elements, restriction enzymes, recombination and repair enzymes. Interestingly, also the bacterial UvrC family of nucleases and Cho (UvrC homologue), both members of the nucleotide excision repair (NER) machinery, belong to the GIY-YIG superfamily. The GIY-YIG domain of LEM-3 belongs to the COG3680 subfamily. Members of this subfamily are only present in metazoa and a few pathogenic bacteria. Their non-pathogenic relatives, however, lack the GIY-YIG domain. Strikingly, the only non-pathogenic bacterial member of the COG3680 family is the polyextremophile Deinococcus radiodurans R1, one of the most radiation-resistant organisms known to date.

By analyzing the domain composition of LEM-3, I got important insights into possible functions of LEM-3. The presence of the GIY-YIG domain suggests a function in DNA repair. Localizing to DNA could be mediated by BAF-1, which is a putative binding partner of the LEM domain. In contrast to the other LEM-domain proteins in *C. elegans*, LEM-3 lacks a transmembrane domain, thus, the protein is probably not an integral component of the nuclear membrane.

LEM-3 is evolutionary highly conserved. A BLAST search revealed that LEM-3 is highly conserved from worm to man. Especially the C-terminus shows high conservation (Figure 8.2). The leucine mutated in *op444* is conserved in all species analysed. This suggests that the residue is important for

the function of the protein.

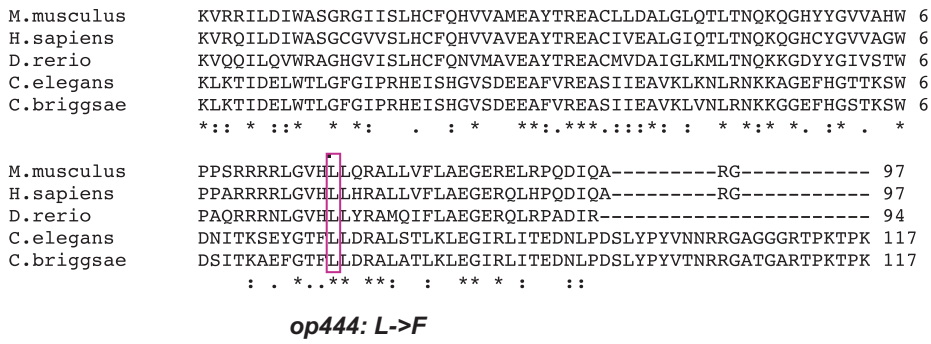


Figure 8.2: **The C-terminus of LEM-3 is evolutionary conserved.** Residues are labelled the following (*) for identical , (:) for conserved and (.) for semiconserved. The residue mutated in *op444* is indicated.

PEST motif

Analysis with http://emboss.bioinformatics.nl/cgi-bin/emboss/epestfind revealed two potential PEST sites in LEM-3: KPTTSSSTSFSSAEDDK (AA 394 to 410) with a PEST score of 17.73 and KEAEVSTPTTVDDGEIR (AA 410 to 426) with a PEST score of 8.95. “Valid” PEST motifs have a score higher than 5. 0. PEST stands for rich in Proline (P), glutamic acid (E), serine (S) and threonine (T) and acts as a signal to target proteins for degradation. The presence of this motif suggests that LEM-3 has a limited intracellular half-life.

Chapter 9

Rescue of the radiation sensitivity of *op444*

WHEN I IDENTIFIED the mutation in *lem-3,* no other alleles of this gene were available. I tried to phenocopy the DNA damage response defect of *op444* animals by RNAi-mediated downregulation of *lem-3* in wild type. By feeding bacteria containing dsRNA corresponding to *lem-3*, I could not confirm the phenotype observed in *op444* mutants. As a next approach, I constructed a *lem-3* rescuing construct for expression in the mutant. The fact that *lem-3* is part of an operon made the choice for the promoter more difficult. That is why, I finally decided to use the promoter of the ubiquitously expressed gene *npp-1* (gift from the Piano lab). *npp-1* encodes part of the nuclear pore complex and expressed in most if not all cells at all developmental stages (Schetter et al., 2006). As some genes show different expression patterns depending on their 3' untranslated region, I decided to use the endogenous *lem-3* UTR. Transgenic lines expressing $P_{npp\text{-}1}$::*lem-*

3_{genomic}::3'$\text{UTR}_{\text{lem-3}}$ were created by biolistic transformation (Praitis et al., 2001). The advantage compared to injection of the plasmid is a higher rate of low-copy stable integration. Three of five independent lines could fully rescue the radiation hypersensitivity of *op444* mutants, two of them are shown in Figure 9.1. Thus, I conclude that the point mutation in *lem-3* is responsible for the phenotype of *op444* mutants.

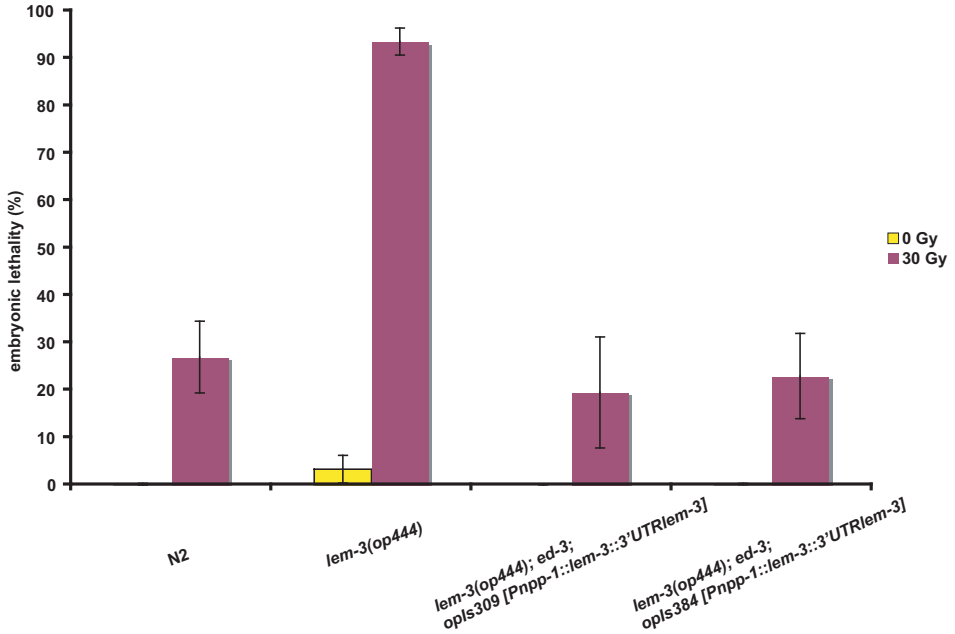


Figure 9.1: **Ectopic expression of LEM-3 rescues the radiation hypersensitivity of *op444***. Two independent transgenic lines expressing $P_{\text{npp-1}}$::*lem-*3_{genomic}::3'$\text{UTR}_{\text{lem-3}}$ rescued the embryonic lethality of *op444* mutants treated with 30 Gy. Data represent the average of two independent experiments ± S. D. for *opIs309* and of three independent experiments ± S. D. for all other strains. The progeny of 10 worms were analysed for each experiment.

To get further insights into the localisation of LEM-3, I created transgenic lines expressing YFP::LEM-3 under the control of the *npp-1* promoter and the endogenous *lem-3* 3' untranslated region. Two lines expressed the con-

struct and rescued the radiation-hypersensitivity (Figure 9.2). Consequently, I analysed the expression profile. The only expression detectable was a foci-like pattern in the embryo (Figure 9.3) that did not change upon irradiation. In some embryos, a weak signal was also found on the plasma membrane. No YFP signal was detected in the nucleus. Foci-like staining is often found for proteins that localise to damaged DNA, like RAD-54 for instance. But as LEM-3-foci were less frequent than DNA damage induced foci and clearly outside the nucleus, I could rule out the possibility that those foci represented active sites of repair. The localisation was unexpected, however, as those transgenic lines fully rescue the phenotype, sufficient amount of LEM-3 is present at the sites of action, which are not necessarily the observed foci.

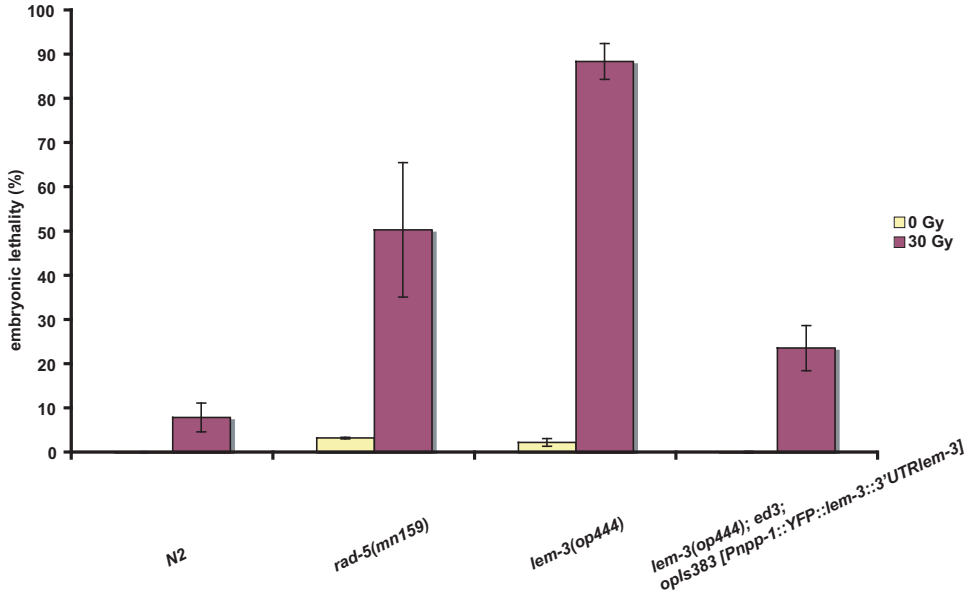


Figure 9.2: **Ectopic expression of YFP::LEM-3 rescues the radiation hypersensitivity of *op444***. Embryonic lethality with and without treatment with 30 Gy was quantified in wild type, *rad-5(mn159)*, *op444* and *op444; ed-3; opIs383*, a transgenic lines expressing $P_{npp\text{-}1}$::YFP*::lem-3*$_{genomic}$::3'UTR$_{lem\text{-}3}$. Data represent the average of three independent experiments ± S. D. The progeny of 10 worms were analysed for each experiment.

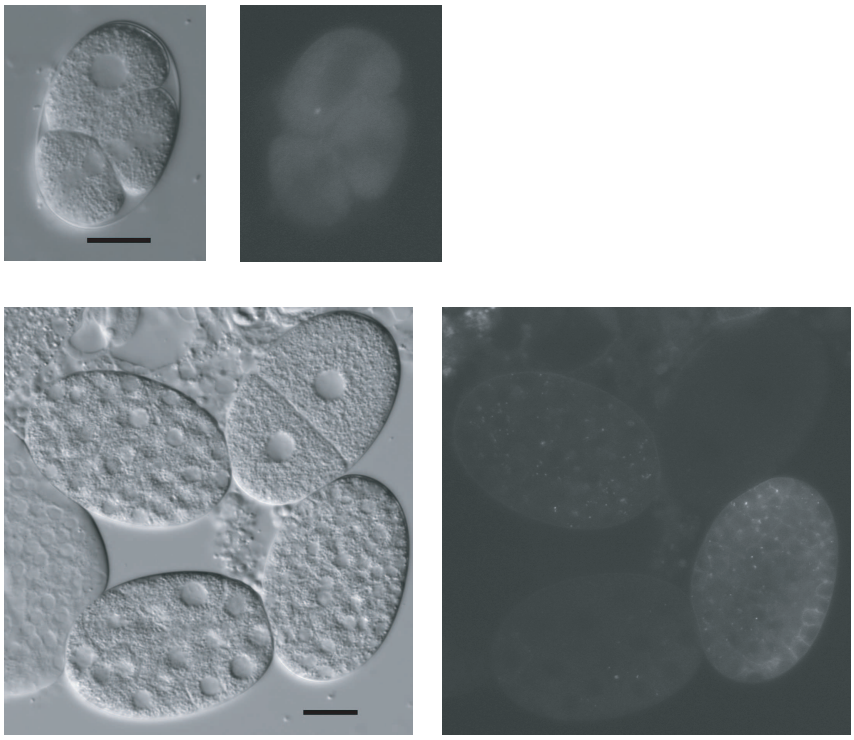


Figure 9.3: **Localisation of LEM-3 in the embryo**. Representative DIC and YFP fluorescence images.

Those foci could also be a staining of the centrosomes in the dividing embryo. To test this possibility, I fixed the YFP-LEM-3 expressing embryos and stained them with an anti-tubulin antibody that stained the mitotic spindle and an anti-GFP antibody that recognized the YFP moiety. The mitotic spindle initiates from the centrosomes, the microtubule organizing centres in animal cells. In this still preliminary experiment, LEM-3 did not co-localize with the mitotic spindle, therefore it is rather unlikely that LEM-3 foci are found on centrosomes.

So far I have not found out what these foci are but the immunostaining showed an important detail, i.e. that the staining is located at the plasma membrane.

Chapter 10

Other *lem-3* alleles

10.1 *rad-1(mn155)*

Three non-allelic radiation-hypersensitive mutations map to chromosome I, *rad-1*, *rad-3* and *rad-8* (Hartman and Herman, 1982). From those, only the molecular identity of *rad-3* has been found so far. It maps to the *C. elegans* ortholog of XPA, a component of the nucleotide excision repair (NER) pathway (Astin et al., 2008). The mapping data of Hartman and Hermann suggest that *rad-3* and *rad-8* both map left of *unc-13*, while *rad-1* maps right of *unc-13*. It is therefore unlikely that *rad-8* is an allele of *lem-3*, as *lem-3* is located right of *unc-13*. Data on WormBase define a mapping position of 6.02 +/- 1.447 for *rad-1*. *lem-3* is not in this suggested interval, thus *rad-1* was unlikely to map to *lem-3*. Interestingly, *rad-1(mn155)* and *lem-3(op444)* animals are both extremely hypersensitive to UV and X-radiation. The similarity of the phenotype provoked me to carry out a complementation test. Surprisingly, *rad-1(mn155)/lem-3(op444)* trans-heterozygotes were hypersensitive to

X-radiation, thus *mn155* is another allele of *lem-3* (Figure 10.1). Sequencing revealed that *rad-1(mn155)* carries a cytosine to thymine transition at base pair 671, which leads to a premature stop codon, R190STOP, in the *lem-3* sequence. The resulting truncated protein lacks the LEM domain and the GIY domain.

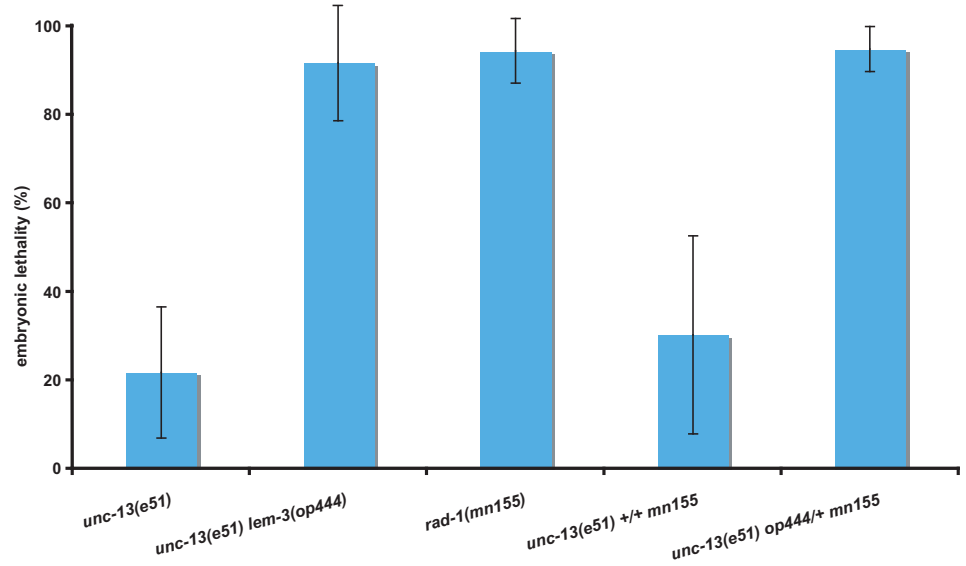


Figure 10.1: ***rad-1(mn155)* fails to complement *lem-3(op444)*.** Embryonic lethality upon treatment with 30 Gy was quantified for *unc-13(e51)*, *unc-13(e51) lem-3(op444)*, *rad-1(mn155)*, *unc-13(e51)* +/+ *rad-1(mn155)* , and *unc-13(e51) lem-3(op444)*/+ *rad-1(mn155).* Average embryonic lethality is shown ± S. D. The progeny of 10 worms were analysed.

10.2 *lem-3(tm3468)*

During the last year another allele became available from the Mitani lab. *tm3468* is a 330bp in-frame deletion allele, which cuts out part of exon 3 (Figure 10.3). The mutant is superficially wild-type. *lem-3(tm3468)* was backcrossed three times and then analysed for its radiation hypersensitivity.

I found that *tm3468* had a much weaker phenotype than the other two alleles. This result is not very surprising, as the translational product of *lem-3(tm3468)* has still all the known functional domains. *tm3468/op444* transheterozygotes showed an intermediate phenotype between the *op444* and the *tm3468* allele (Figure 10.2)

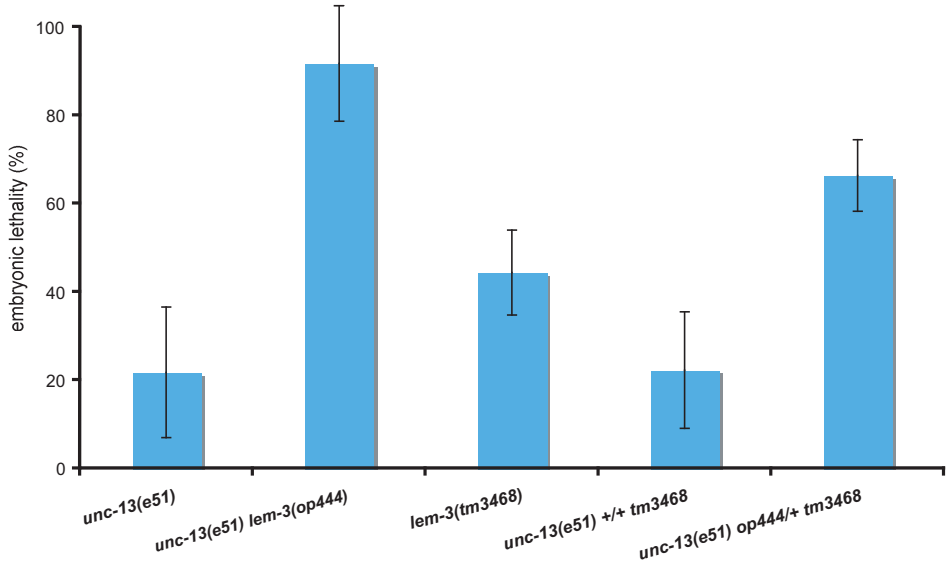


Figure 10.2: ***tm3468* is a weaker allele of *lem-3*.** Embryonic lethality upon treatment with 30 Gy was quantified for *unc-13(e51)*, *unc-13(e51) lem-3(op444)*, *lem-3(tm3468)*, *unc-13(e51)* +/+ *lem-3(tm3468)* , and *unc-13(e51) lem-3(op444)*/+ *lem-3(tm3468).* Average embryonic lethality is shown ± S. D. The progeny of 10 worms were analysed.

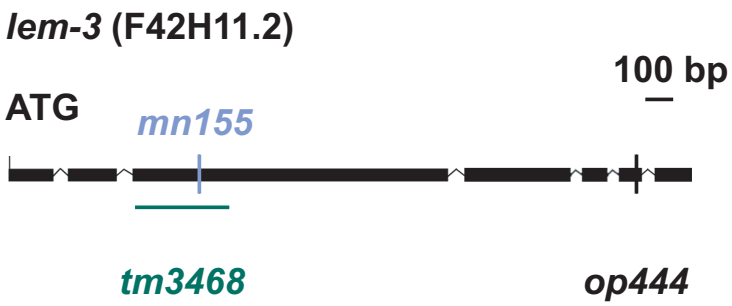


Figure 10.3: **Mutant alleles of *lem-3***. mSplicer prediction of the *lem-3* gene structure. Exons are depicted as boxes, the point mutations *op444* (black) and *mn155* (blue) are shown as vertical lines. The *tm3468* in-frame deletion (green) is indicated with a horizontal line.

Chapter 11

Defects in irradiated *lem-3(op444)* embryos

AS I COULD NOT FIND any defect in cell cycle arrest nor in apoptosis, I wanted to investigate why the embryos finally die. Therefore, I irradiated *op444* hermaphrodites, dissected the embryos from the irradiated mothers, fixed them and stained the DNA with 4´,6-Diamidino-2-phenylindol (DAPI). The staining revealed the presence of abnormal DNA structures, such as micronuclei and anaphase bridges (Figure 11.1). To further corroborate the result, I crossed *op444* animals with a transgenic line carrying a GFP-labeled histone H2B. This marker protein faithfully labels chromatin and allows live-imaging of dividing embryos. Movies of irradiated *op444* embryos revealed that the first cell division was indistinguishable from wild type. The P0 blastomere divided unequally, giving rise to the AB blastomere and the smaller P1 blastomere. Then the AB blastomere divided before the P1 cell, which did not differ between wild type and *op444* animals either. During the divi-

sions of the AB and the P1 cells chromatin did not segregate faithfully. DNA was observed in the cytoplasm, some of it formed micronuclei or disappeared. Representative images are shown in Figure 11.3.

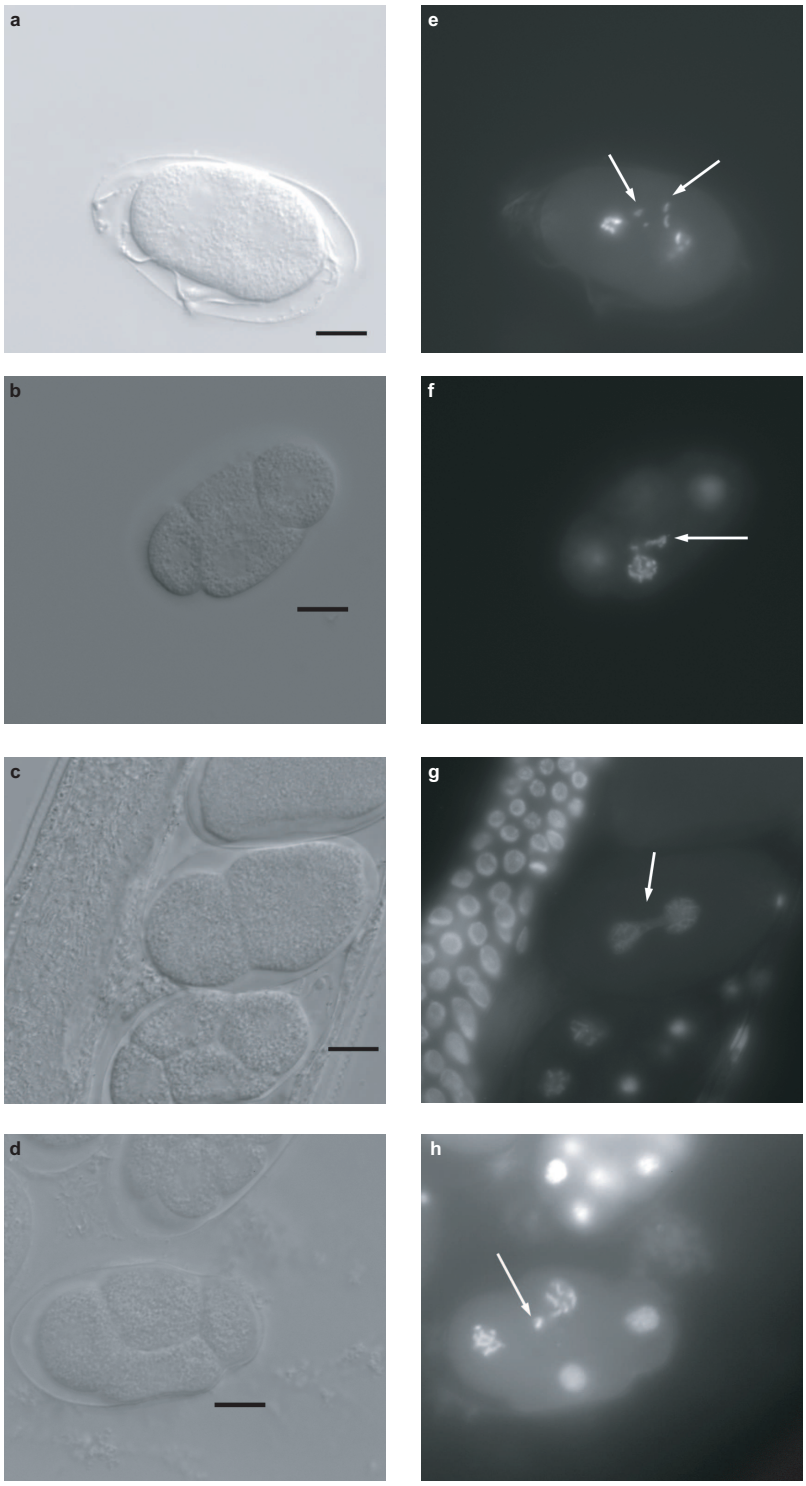
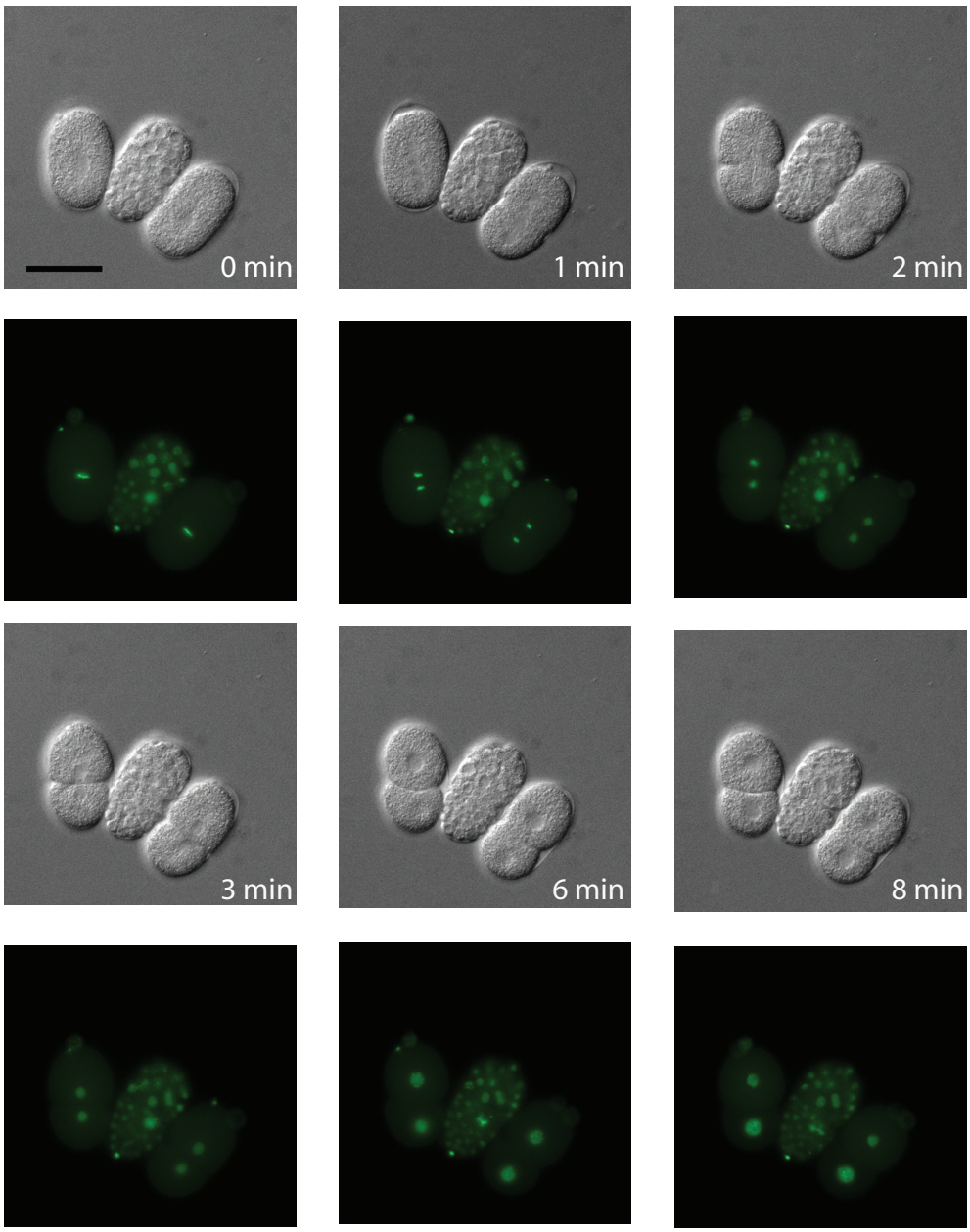


Figure 11.1: **Defects in irradiated *op444* embryos**. Synchronized adult worms were irradiated with 30 Gy. Embryos were dissected from the gonads and fixed. Chromatin was stained with 4´,6-Diamidino-2-phenylindol (DAPI). (a-d) DIC pictures. Arrows in (e), (f), and (h) indicate abnormally localised DNA. The arrow in (g) indicates an anaphase bridge. Representative images are shown. Size bar is $10\mu m$.


0 min
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8 min

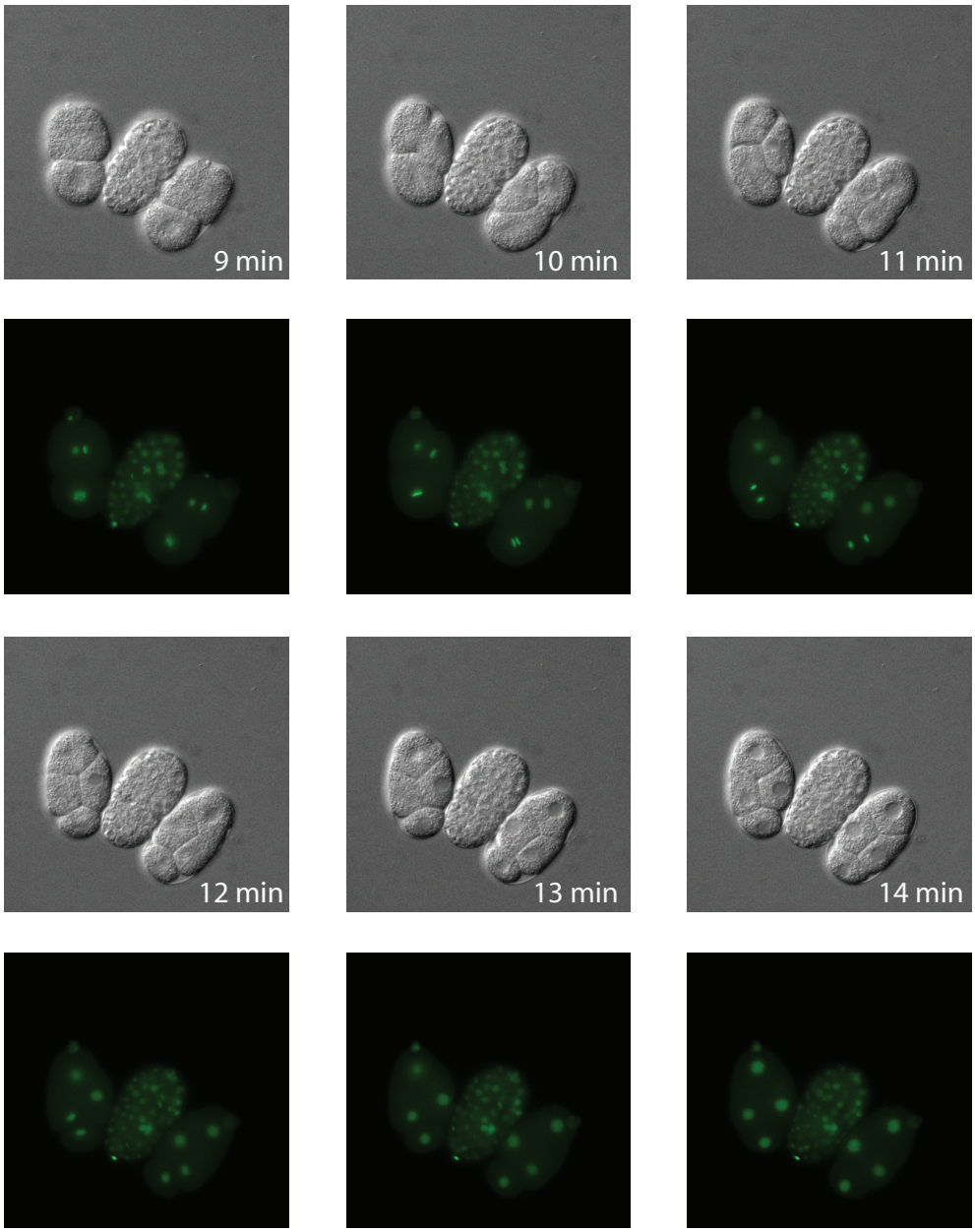
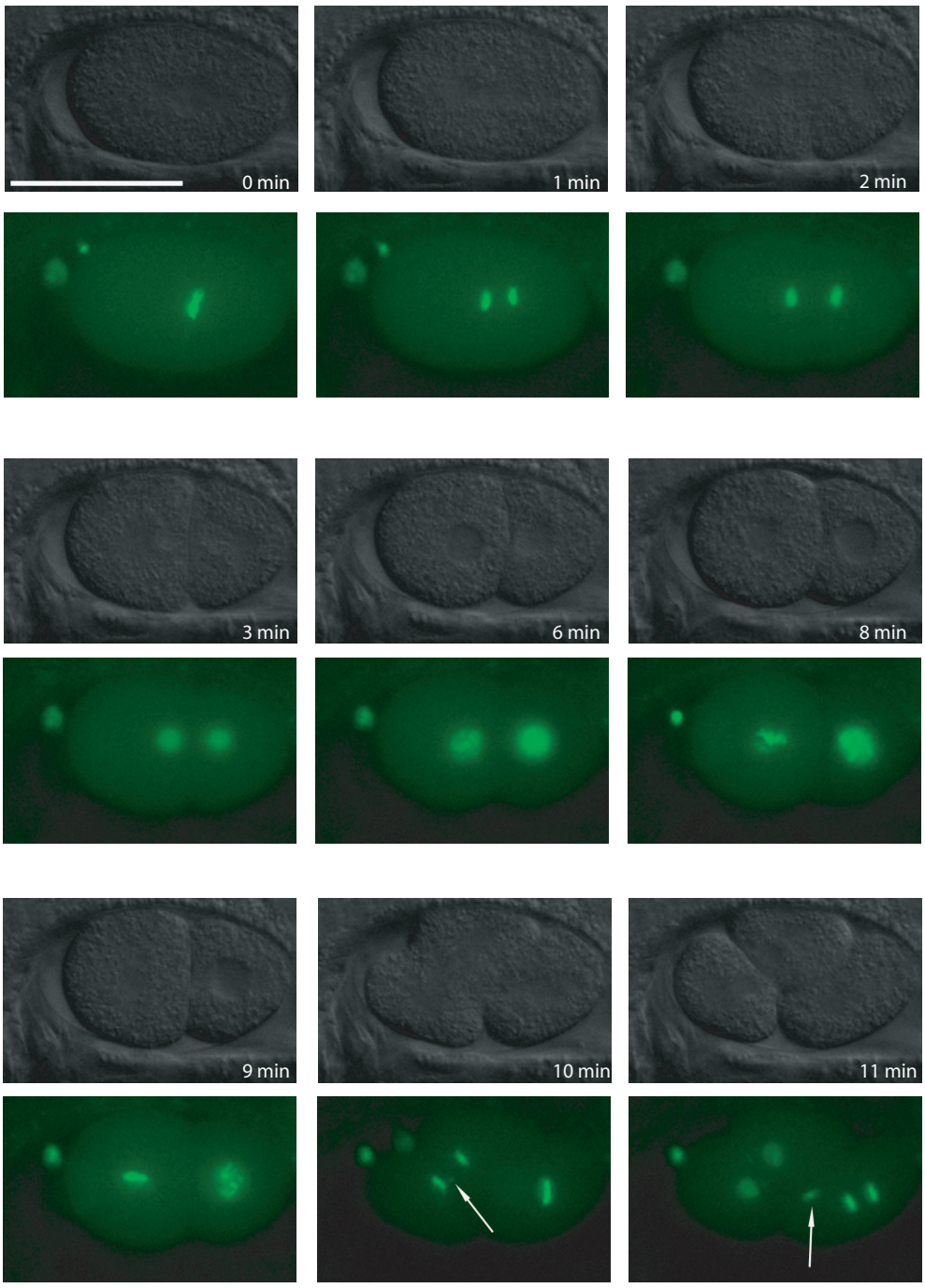


Figure 11.2: **Time lapse analysis of irradiated embryos**. Embryos carrying H2B::GFP were irradiated with 30 Gy. Upper panels Nomarski images, lower pannels GFP. Size bar is $30\mu m$.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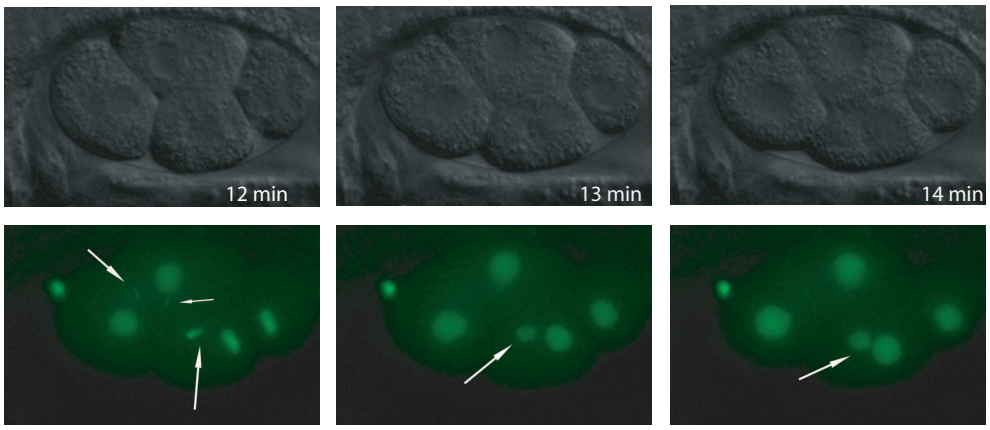

Figure 11.3: **Time lapse analysis of irradiated *op444* embryos**. *op444* embryos carrying H2B::GFP were irradiated with 30 Gy. Upper panels Nomarski images, lower pannels GFP. Abnormal chromosome structures are indicated with arrows. Size bar is $30\mu m$.

Part III

Materials and Methods

Strains and general procedures

N2 Bristol strain (Brenner, 1974)was used as wild-type strain in all experiments. For mapping experiments the polymorphic strain CB4856 was used. All strains were kept on NGM agar plates seeded with Escherichia coli OP50 at 20°C unless otherwise stated. All strains were provided by the Caenorhabditis Genetics Center (CGC) unless otherwise stated. *lem-3(tm3468),* which was kindly provided by the Mitani lab.

LG I: *dpy-5(e61), unc-13(e51), dpy-24(s71), xpa-1(ok698), rad-1(mn155), lem-3 (op444), lem-3(tm3468)*

LG II: *rol-6(e187)*

LG III: *unc-32(e189), rad-5/clk-2(mn159)*

LG IV: *unc-5(e53), him-6*

LG V: *dpy-11(e224)*

LG X: *lon-2(e678)*

Transgenic lines:

opIs257 [RAD-54::YFP] made by Dr. Lilli Stergiou (manuscript in preparation)

AZ212*: unc-119(ed3) ruIs32[unc-119(+) pie-1*::GFP::H2B]

Ethyl methane sulfonate (EMS) based screen

Synchronized L4-stage wild-type worms (Brenner, 1974) were subjected to 50mM EMS (M0880 Sigma-Aldrich) and incubated on a nutator at 20°C for 4 hours. They were washed four times with M9 and transferred to a seeded

plate. Worms were allowed to recover overnight at 15°C. The F1 generation was transferred to new plates and let lay eggs. The number of F1 worms per plate was limited to fifty. Fifty Worms of the F2 generation were singled out at L4 stage to NGM agar plates ("backup plates"). The next day those worms that had laid eggs were transferred to 24-well NGM agar plates ("screening plates") and subjected to X-radiation. First, 10 Gy were used to inflict DNA damage, however, in order to find more candidates later on the dose was increased to 60 Gy. Sterile worms or egg-laying defective worms were not transferred to a screening plate but excluded. The next day the worms were removed from the 24-well plates. The day after the plates were examined for lethality after irradiation. Those candidates whose progeny hatched on the "backup plates" but died upon irradiation on the "screening plates" were subjected to further analysis.

Irradiation

An Isovolt Titan 160 with Isovolt 160 M2/0.4 - 3.0 (Seifert) and a Stratalinker UV crosslinker, model 1800 (Stratagene) were used to deliver the indicated doses.

Microscopy

For preparation of germlines or embryos, worms were dissected in M9 buffer. For observation of whole worms, they were put on a 2% agarose (dissolved in M9) pad and anasthetized with levamisole (20mM). For microscopic observation a Leica DMRA2 microscope equipped with DIC (Nomarski) optics and

epifluorescence was used. Images were acquired with an ORCA-ER digital CCD camera and were processed with Openlab software (Improvision). For time lapse microscopy, an Olympus BX61 was used equipped with Retiga 2000R camera. Pictures were processed using Openlab software (Improvision). Worms were dissected 1.5 to 5 hours after irradiation and dissected on a coverslip, the embryos were then put on a 2% agarose pad. Images were taken every 20 sec at 2x binning.

Germline apoptosis

Synchronized animals were irradiated with 60 Gy 12 hours post L4 stage. Apoptotic corpses, which appeared as refractive, rounded structures, were quantified in the meiotic zone of the germ line at indicated time points, as described in Gumienny et al., 1999.

Cell cycle arrest

Synchronized animals were irradiated at L4 stage. Germ lines were dissected at indicated time points. Images were taken using an ORCA-ER digital CCD camera. The diameters of 10 nuclei (in focus) were measured and the average diameter of nuclei per germ line was calculated using ImageJ 1.40g software (Wayne Rasband, http://rsb.info.nih.gov/ij).

Immunofluorescence

Embryos were dissected from the gonads using a 20 Gauche syringe in a drop of M9 onto a diagnostic microscopy slide (30-2066A-Brown-CE24 3 squares

14 mm x 14 mm with bars by CEL-LINE). They were squashed with a cover slip, which was placed crossways onto the slide, and immediately frozen in liquid nitrogen. The cover slip was popped off to break the egg shell. Fixation was done with methanol for 20 min at -20°C. The slides were washed with PBS for 5 min. Blocking was performed in PBS-Tween (0.2%) 1% BSA (Roche) for 20 min. The first antibody was diluted in blocking solution and applied over night at 4°C in a humid chamber. The slides wer then washed with PBS-Tween twice for 10 min. They were incubated with the secondary anitbody and 4',6-diamidino-2-phenylindole (DAPI) for 45 min at 37°C in a humid chamber. Washing was performed as described above. Fluoro-Guard Antifade reagent (Bio Rad) was used as mounting medium.

Reagents: rabbit anti-GFP ab6556 (Abcam) was a gift from the Gotta lab, mouse anti-α-tubulin TT6074 (Sigma) was a gift from Dr. Alex Hajnal, rabbit anti-α-tubulin ab18251 (Abcam), secondary antibodies were Alexa fluor 488 or 594 antibodies (Molecular Probes), DAPI (Molecular probes).

RAD-54::YFP foci

Synchronized young adults were irradiated 12 hours post L4 stage. Germ lines were dissected 3 hours after treatment. Images were acquired with a Leica DMRA2 microscope equipped with an ORCA-ER digital CCD camera. Foci were analysed with ImageJ 1.40g software (Wayne Rasband, http://rsb.info.nih.gov/ij).

Transgenic lines

Transgenic lines were obtained by microparticle bombardment in a Biolistic PDS-1000 (Bio-Rad) transformation, as described in Praitis et al., 2001.

DNA constructs

PCR amplification was done with Phusion High-Fidelity DNA Polymerase (Finnzymes). Oligonucleotide primers were synthesized by Microsynth.

***npp-1* promoter:**

For subcloning of the ***npp-1* promoter** into pJET (GeneJet PCR cloning Kit by Fermentas), the following primers were used (shown in 5' – 3' direction):

Pnpp-1Sbf_fw

GATCCCTGCAGGTTATTGGTGTCATTTCGGTGATTATGATTG

Pnpp-1Asc_rev

CCATGGCGCGCCTTCGCTGAAAACAAACGATTTTTAAGAGAAATG

***lem-3* genomic plus 3' UTR:**

For subcloning *lem-3* genomic (2551 bp) plus 3'UTR (990 bp) into pJET (GeneJet PCR cloning Kit by Fermentas), the following primers were used (shown in 5' – 3' direction):

3lem3AscIfw

GATCGGCGCGCCATGCCTCCAAACGGAGCAATCACCACGA

3lem3ApaIrev

CTGAGGGCCCAGCCAATTCAACCGACTAATAAGGTAGATT

YFP

A AscI-YPF-AscI cassette was obtained from Dr. Lukas Neukomm and inserted into the plasmid.

Part IV

Discussion and Outlook

THIS STUDY shows that a forward genetic screen in *C. elegans* can still identify novel components in the DNA damage response pathways. When I started this project, the main criticism I encountered was, first, that I would only find already known factors and, second, that my study would reveal solely worm-specific factors. Finally, I can definitely rule out the first as this is the first report connecting LEM-3 to a function in the DNA damage responses. Whether LEM-3 also acts in the DNA damage responses of higher eukaryotes or is a worm-specific component, remains to be determined. One argument in favour of a similar role in higher eukaryotes is the conservation of the protein sequence, especially of the GIY-YIG containing C-terminus.

rad-1(mn155) **is allelic to** ***lem-3(op444)***

This study is the first report showing that a mutation in *lem-3* causes the *rad-1* phenotype. The mutation is an early stop and deletes the C-terminus of the protein. The mutation is likely to be a strong loss-of-function mutation if not even a null mutation. Interestingly, *op444* and *rad-1(mn155)* animals show the same high degree of radiation sensitivity (Figure 10.1) demonstrating that the residue mutated in *op444* animals must fulfil a critical if not essential role for the function of the protein. Another mutant allele *tm3468,* which is an in-frame deletion, does not display a radiation sensitivity as profound as the mutations *op444* or *mn155* (Figure 10.2).

Cell cycle arrest response and apoptosis in ***lem-3(op444)*** **animals**

Upon irradiation, mitotic germ cells of *lem-3(op444)* mutants show normal cell cycle arrest (Figures 6.5 and 6.6). This halt in progression is transient,

as normal cycling of the nuclei is observed after some time. This shows that unlike the components of the 9-1-1 pathway or RAD-5/CLK-2, LEM-3 does not play a role in the G2/M cell cycle arrest response. CEP-1, the *C. elegans* homolog of p53, has been shown to play a pivotal role in apoptosis. Failures to activate p53 upon irradiation lead to a lack of apoptotic corpses in the pachytene zone. *lem-3(op444)* mutants initiate apoptosis in a wild-type manner (Figure 6.4) underscoring again the result that the upstream signalling pathways and the ability to carry out a proper apoptotic response are not impaired.

Double-strand break repair in *lem-3(op444)* mutants

Double-strand breaks in the germ line of *C. elegans* are removed by homologous recombinational repair. The alternative pathway, NHEJ, plays a role in non-dividing somatic tissues of the worm only but not in the germ line (Clejan et al., 2006). The formation of RAD-54 foci in irradiated mitotic nuclei indicate that double-strand break repair by homologous recombination is initiated (Figure 6.8). Those foci also disappear at later time points except for a minor fraction of the nuclei, which fail to remove RAD-54 from chromatin (Figure 6.9). Those RAD-54 foci retaining nuclei appear still enlarged suggesting that they remain in an arrested state. They occur in wild-type and *op444* germ lines and are presumably cells that have high numbers of DNA lesions that exceed the repair capacity of the cell. It can be speculated that the future fate of these cells will be death by apoptosis due to high amounts of damage. If the repair activity in *op444* mutants were non-functional, all cells would still show RAD-54 foci. This demonstrates DNA double-strand breaks

are removed by homologous recombinational repair in *op444* mutants.

lem-3 and meiosis

None of the *lem-3* alleles analysed showed a high incidence of X0 males or high embryonic lethality in the absence of a genotoxic insult. This shows that chromosome segregation is not defective, as random segregation of the X chromosome would cause a male phenotype (X0 are males, XX and XXX are hermaphrodites) and random segregation of autosomes would cause embryonic lethality. The absence of F1 embryonic lethality in non-irradiated *op444* further suggests that there is no major defect in meiosis, which is consistent with the finding that initiation of DSB repair appears to be wildtype. During DSB repair in meiosis and in the DNA damage response, a lot of factors are shared. If DSB repair were defective, *op444* mutants would show embryonic lethality due to problems in repairing breaks that arise during homologous recombination in meiosis. RNAi of *rad-51* (Gartner et al., 2000; Boulton et al., 2002) and *rad-54* (Boulton et al., 2002), as well as mutants of the *C. elegans* homologs of BRCA1 and BARD1 (Boulton et al., 2004) display phenotypes during meiosis like an increase in apoptotic corpses in the absence of exogenous DNA damage and a high incidence of males. None of these phenotypes has been observed in *op444* animals indicating that meiosis is not impaired. Thus, I conclude that DSB repair is functional and does not account for the drastic embryonic lethality upon irradiation.

Repair activities in oocytes and sperm?

Although a DNA damage phenotype in oocytes alone has never been observed before, it might be possible that some repair activities are restricted to oocytes only and that residual damage might be removed in a final step before fertilization. In case of residual DSB, chromosome fragmentation would be observed in oocytes as for irradiated *mrt-2* (Clejan et al., 2006). This assay has not been performed so far and will shed more light into the potential role of oocytes in the radiation sensitivity of *op444* animals. Intact chromosomes, however, do not rule out that there are still lesions present, as simple DNA adducts or bulky lesions would not cause chromosome breaks but pose a severe problem to the replication machinery in the future embryo. Repair activity in sperm is a neglected topic in *C. elegans* as sperm DNA is highly compact and condensed and hence not accessible to DNA damage markers. There is probably no cell cycle arrest as the cells are not dividing anymore. Apoptosis has not been observed in sperm (Gumienny et al., 1999; Gartner et al., 2000). More sophisticated experiments will be required to determine whether a defect in irradiated sperm accounts for the radiation-sensitivity of *op444* mutants.

Localisation of LEM-3

Consistent with the lack of germ line phenotypes, no expression of LEM-3 has been detected in this tissue. LEM-3 localises to foci in the embryos (Figure 9.3), which are distinct from centrosomes. The exact nature of these foci has yet to be discovered. One possibility is that those structures are the remnants of the central spindle. At the onset of anaphase, microtubules pull the chromosomes into a poleward direction, non-kinetochore microtubules

form a bundled structure and assemble as part of the central spindle complex (also referred to as midbody). BIR-1 (Speliotes et al., 2000), which is the *C. elegans* ortholog of survivin, dynamin (Thompson et al., 2002), ZEN-4 (Guse et al., 2005), and SPD-1 (Verbrugghe and White, 2004), for instance, show such a foci-like staining at the central zone of the spindle. Co-localisation experiments are required to support the hypothesis that LEM-3 localizes to the central spindle.

LEM-3 a GIY-YIG family member

So far, no function has been shown for LEM-3 in *C. elegans*. This study provides the first evidence that LEM-3 plays a pivotal role in DNA integrity maintenance after DNA damage. As no function in checkpoint signalling nor in programmed cell death has been found, LEM-3 appears to act in another DNA damage response, presumably in DNA repair. In support of the hypothesis that LEM-3 is a repair enzyme, is the fact that it contains a COG3680 domain, which has been reported to be a member of the UvrC family of GIY-YIG nucleases (Dunin-Horkawicz et al., 2006). The GIY-YIG domain is found in enzymes involved in DNA repair and recombination. Two famous GIY-YIG family members are the endonucleases UvrC and Cho, which play a pivotal role in bacterial NER. UvrC cuts 3' and 5' of the DNA lesion, while Cho can only cut at the 3' site (Moolenaar et al., 2002). As the GIY-YIG domain has been shown to be responsible for the 3' incision only (Truglio et al., 2005), LEM-3 may need a partner to cut out the stretch of DNA containing a lesion. The presence of an ankyrin domain, which mediates protein-protein interactions, could be crucial for such a binding partner. If LEM-3 acted as an

endonuclease, the purified protein should be able to cut DNA *in vitro* whereas a mutation in the catalytic site should abolish this activity.

LEM-3, a LEM-domain protein

The LEM domain is found in inner nuclear membrane proteins and nucleoplasmic proteins (Gruenbaum et al., 2005). LEM-3 does not have a transmembrane domain, which makes it unlikely to be part of the nuclear membrane. In contrast to LEM-like domains, the LEM domain itself cannot bind DNA directly (Cai et al., 2001). Hence, interaction of LEM-3 with DNA is probably mediated by other proteins. One candidate is the small conserved protein BAF-1, which binds to the LEM domain and to dsDNA sequence-independently. Null-mutations in *Cebaf-1* are lethal. Embryos die at about 100-cell stage with abnormally condensed chromatin and anaphase bridges. The existence of a temperature-sensitive allele might allow double mutant analysis, which will reveal whether they act together in a common pathway. Alternatively, localisation of LEM-3 to DNA could also be dependent on an ankyrin-repeat-mediated interaction with a DNA-binding protein.

The function of LEM-3 in the *C. elegans* embryos

Although LEM-3 is a highly conserved protein, no functional analysis has been performed so far. In a large scale RNAi screen, the human LEM-3 ortholog ANKRD41 was found to protect cells from low doses of taxol (Whitehurst et al., 2007). This might provide a first evidence that LEM-3 is involved in spindle stability. Spindle defects have been observed in *rad-5/clk-2* embryos at restrictive temperature. But those spindle defects in embryos are

independent of DNA damage.

A different role for LEM-3 is suggested by this study. LEM-3 is shown to be a crucial component for the survival of radiation-exposed embryos. The absence of a role in cell cycle arrest and apoptosis in the germ line suggests that it is involved in another DNA damage response pathway, possibly DNA repair. The COG3680 domain found in LEM-3 belongs to the UvrC-like branch of the GIY-YIG domains (Dunin-Horkawicz et al., 2006). Given that the major nucleases involved in bacterial and eukaryotic NER have already been identified, Dunin and Coworkers speculate that in analogy to Cho, COG3680 proteins may act in a specialized repair pathway to remove bulky lesions. The NER pathway is a highly versatile type of repair. It can repair many structurally unrelated types of DNA damage (Truglio et al., 2005). One of those are helix-distorting lesions, which pose a severe problem to DNA replication. UV-C is known to cause such bulky DNA lesions (Hoeijmakers, 2001). Ionizing irradiation, such as X-rays, does not exclusively cause double-strand breaks, but also in a direct or indirect way a myriad of other types of damage. Some types of lesions found in gamma-irradiated DNA are removed by the bacterial UvrABC system (Roldán-Arjona and Sedgwick, 1996) or the human NER pathway (Satoh et al., 1993). Cyclopurine deoxynucleosides, which are found in irradiated DNA, are shown to impair primer extension by DNA polymerases. Their removal specifically requires the NER pathway (Kuraoka et al., 2000). Double-strand breaks probably do not cause the radiation-induced high embryonic lethality in *lem-3(op444)* mutants. The high embryonic lethality might be caused by the failure to remove bulky lesions from DNA. They do not pose a problem as long as there is no fast DNA replication going on; that is why *lem-3* appears to have no phenotypes in the

germ line. In the embryo, however, requirements are different. Replication needs to be fast as the timing of cell division is developmentally programmed. The repair mechanism has to act quickly on the bulky lesions, so that the DNA polymerase can replicate the DNA.

In conclusion, my PhD thesis has led to the discovery of a novel factor in the DNA damage response pathways. It has revealed that LEM-3, a protein of so far uncharacterized function, protects *C. elegans* embryos from radiation-induced lethality. I also found that the radiation-sensitive phenotype of *rad-1*, a mutant whose molecular identity has been enigmatic so far, is caused by a mutation in *lem-3*. LEM-3 presumably acts as a repair endonuclease and removes DNA lesions that obstruct DNA synthesis in the developing embryo.

Bibliography

A

Ahmed, S., Alpi, A., Hengartner, M. O., and Gartner, A. (2001). C. elegans rad-5/clk-2 defines a new dna damage checkpoint protein. *Curr Biol*, 11(24):1934–44.

Ahmed, S. and Hodgkin, J. (2000). Mrt-2 checkpoint protein is required for germline immortality and telomere replication in c. elegans. *Nature*, 403(6766):159–64.

Astin, J. W., O'Neil, N. J., and Kuwabara, P. E. (2008). Nucleotide excision repair and the degradation of rna pol ii by the caenorhabditis elegans xpa and rsp5 orthologues, rad-3 and wwp-1. *DNA Repair*, 7(2):267–80.

B

Baehrecke, E. H. (2002). How death shapes life during development. *Nat. Rev. Mol. Cell Biol.*, 3(10):779–87.

Boulton, S., Martin, J., Polanowska, J., Hill, D., Gartner, A., and Vidal, M. (2004). Brca1/bard1 orthologs required for dna repair in caenorhabditis elegans. *Current Biology*, 14(1):33–39.

Boulton, S. J., Gartner, A., Reboul, J., Vaglio, P., Dyson, N., Hill, D. E., and Vidal, M. (2002). Combined functional genomic maps of the c. elegans dna damage response. *Science*, 295(5552):127–31.

Brauchle, M., Baumer, K., and Gönczy, P. (2003). Differential activation of the dna replication checkpoint contributes to asynchrony of cell division in c. elegans embryos. *Curr Biol*, 13(10):819–27.

Brenner, S. (1974). The genetics of caenorhabditis elegans. *Genetics*, 77(1):71–94.

Brenner, S. (2003). Nobel lecture. nature's gift to science. *Biosci Rep*, 23(5-6):225–37.

Buss, R. R., Sun, W., and Oppenheim, R. W. (2006). Adaptive roles of programmed cell death during nervous system development. *Annu. Rev. Neurosci.*, 29:1–35.

C

Cai, M., Huang, Y., Ghirlando, R., Wilson, K. L., Craigie, R., and Clore, G. M. (2001). Solution structure of the constant region of nuclear envelope protein lap2 reveals two lem-domain structures: one binds baf and the other binds dna. *EMBO J*, 20(16):4399–407.

Cheung, I., Schertzer, M., Rose, A., and Lansdorp, P. M. (2002). Disruption of dog-1 in caenorhabditis elegans triggers deletions upstream of guanine-rich dna. *Nat. Genet.*, page 5.

Chin, G. M. and Villeneuve, A. M. (2001). C. elegans mre-11 is required for meiotic recombination and dna repair but is dispensable for the meiotic g(2) dna damage checkpoint. *Genes Dev*, 15(5):522–34.

Church, D. L., Guan, K. L., and Lambie, E. J. (1995). Three genes of the map kinase cascade, mek-2, mpk-1/sur-1 and let-60 ras, are required for meiotic cell cycle progression in caenorhabditis elegans. *Development*, 121(8):2525–35.

Clejan, I., Boerckel, J., and Ahmed, S. (2006). Developmental modulation of nonhomologous end joining in caenorhabditis elegans. *Genetics*, 173(3):1301–17.

Conradt, B. and Horvitz, H. R. (1998). The c. elegans protein egl-1 is required for programmed cell death and interacts with the bcl-2-like protein ced-9. *Cell*, 93(4):519–29.

D

Deng, X., Hofmann, E. R., Villanueva, A., Hobert, O., Capodieci, P., Veach, D. R., Yin, X., Campodonico, L., Glekas, A., Cordon-Cardo, C., Clarkson, B., Bornmann, W. G., Fuks, Z., Hengartner, M. O., and Kolesnick, R. (2004). Caenorhabditis elegans abl-1 antagonizes p53-mediated germline apoptosis after ionizing irradiation. *Nat. Genet.*, 36(8):906–12.

Derry, W. B., Putzke, A. P., and Rothman, J. H. (2001). Caenorhabditis elegans p53: role in apoptosis, meiosis, and stress resistance. *Science*, 294(5542):591–5.

Dunin-Horkawicz, S., Feder, M., and Bujnicki, J. M. (2006). Phylogenomic analysis of the giy-yig nuclease superfamily. *BMC Genomics*, 7:98.

E

Ellis, H. M. and Horvitz, H. R. (1986). Genetic control of programmed cell death in the nematode c. elegans. *Cell*, 44(6):817–29.

Encalada, S. E., Martin, P. R., Phillips, J. B., Lyczak, R., Hamill, D. R., Swan, K. A., and Bowerman, B. (2000). Dna replication defects delay cell division and disrupt cell polarity in early caenorhabditis elegans embryos. *Dev Biol*, 228(2):225–38.

G

Garcia-Muse, T. and Boulton, S. J. (2005). Distinct modes of atr activation after replication stress and dna double-strand breaks in caenorhabditis elegans. *EMBO J*, 24(24):4345–55.

Gartner, A., Boag, P. R., and Blackwell, T. K. (2008). Germline survival and apoptosis. *WormBook : the online review of C elegans biology*, pages 1–20.

Gartner, A., Milstein, S., Ahmed, S., Hodgkin, J., and Hengartner, M. O. (2000). A conserved checkpoint pathway mediates dna damage–induced apoptosis and cell cycle arrest in c. elegans. *Mol Cell*, 5(3):435–43.

Glucksmann, A. (1951). Cell deaths in normal vertebrate ontogeny. *Biol. Rev.*, 26:5986.

Greiss, S., Hall, J., Ahmed, S., and Gartner, A. (2008). C. elegans sir-2.1 translocation is linked to a proapoptotic pathway parallel to cep-1/p53 during dna damage-induced apoptosis. *Genes Dev*, 22(20):2831–2842.

Gruenbaum, Y., Margalit, A., Goldman, R. D., Shumaker, D. K., and Wilson, K. L. (2005). The nuclear lamina comes of age. *Nat. Rev. Mol. Cell Biol.*, 6(1):21–31.

Gumienny, T. L., Lambie, E., Hartwieg, E., Horvitz, H. R., and Hengartner, M. O. (1999). Genetic control of programmed cell death in the caenorhabditis elegans hermaphrodite germline. *Development*, 126(5):1011–22.

Guse, A., Mishima, M., and Glotzer, M. (2005). Phosphorylation of zen-4/mklp1 by aurora b regulates completion of cytokinesis. *Curr Biol*, 15(8):778–86.

H

Hartman, P. S. and Herman, R. K. (1982). Radiation-sensitive mutants of caenorhabditis elegans. *Genetics*, 102(2):159–78.

Hengartner, M. O., Ellis, R. E., and Horvitz, H. R. (1992). Caenorhabditis elegans gene ced-9 protects cells from programmed cell death. *Nature*, 356(6369):494–9.

Hengartner, M. O. and Horvitz, H. R. (1994). C. elegans cell survival gene ced-9 encodes a functional homolog of the mammalian proto-oncogene bcl-2. *Cell*, 76(4):665–76.

Heyer, W.-D., Li, X., Rolfsmeier, M., and Zhang, X.-P. (2006). Rad54: the swiss army knife of homologous recombination? *Nucleic Acids Res*, 34(15):4115–25.

Hirsh, D., Oppenheim, D., and Klass, M. (1976). Development of the reproductive system of caenorhabditis elegans. *Dev Biol*, 49(1):200–19.

Hodgkin, J., Horvitz, H., and Brenner, S. (1979). Nondisjunction mutants of the nematode caenorhabditis elegans. *Genetics*, 91(1):67–94.

Hoeijmakers, J. H. (2001). Genome maintenance mechanisms for preventing cancer. *Nature*, 411(6835):366–74.

Hofmann, E. R., Milstein, S., Boulton, S. J., Ye, M., Hofmann, J. J., Stergiou, L., Gartner, A., Vidal, M., and Hengartner, M. O. (2002). Caenorhabditis elegans hus-1 is a dna damage checkpoint protein required for genome stability and egl-1-mediated apoptosis. *Curr Biol*, 12(22):1908–18.

Holway, A. H., Kim, S.-H., Volpe, A. L., and Michael, W. M. (2006). Checkpoint silencing during the dna damage response in caenorhabditis elegans embryos. *J Cell Biol*, 172(7):999–1008.

Horvitz, H. R. (1999). Genetic control of programmed cell death in the nematode caenorhabditis elegans. *Cancer Res*, 59(7 Suppl):1701s–1706s.

Horvitz, H. R. and Sternberg, P. W. (1982). Nematode postembryonic cell lineages. *J Nematol*, 14(2):240–8.

Houten, B. V., Eisen, J. A., and Hanawalt, P. C. (2002). A cut above: discovery of an alternative excision repair pathway in bacteria. *Proc Natl Acad Sci USA*, 99(5):2581–3.

J

Jacobson, M. D., Weil, M., and Raff, M. C. (1997). Programmed cell death in animal development. *Cell*, 88(3):347–54.

Jiricny, J. (2006). The multifaceted mismatch-repair system. *Nat. Rev. Mol. Cell Biol.*, 7(5):335–346.

K

Kimble, J. and Crittenden, S. L. (2007). Controls of germline stem cells, entry into meiosis, and the sperm/oocyte decision in caenorhabditis elegans. *Annu Rev Cell Dev Biol*, 23:405–33.

Kimble, J. and Hirsh, D. (1979). The postembryonic cell lineages of the hermaphrodite and male gonads in caenorhabditis elegans. *Dev Biol*, 70(2):396–417.

Kimble, J. E. and White, J. G. (1981). On the control of germ cell development in caenorhabditis elegans. *Dev Biol*, 81(2):208–19.

Kuraoka, I., Bender, C., Romieu, A., Cadet, J., Wood, R. D., and Lindahl, T. (2000). Removal of oxygen free-radical-induced 5',8-purine cyclodeoxynucleosides from dna by the nucleotide excision-repair pathway in human cells. *Proc Natl Acad Sci USA*, 97(8):3832–7.

L

Lee, M. S. and Craigie, R. (1994). Protection of retroviral dna from autointegration: involvement of a cellular factor. *Proc Natl Acad Sci USA*, 91(21):9823–7.

Lee, M. S. and Craigie, R. (1998). A previously unidentified host protein protects retroviral dna from autointegration. *Proc Natl Acad Sci USA*, 95(4):1528–33.

Lettre, G. and Hengartner, M. O. (2006). Developmental apoptosis in c. elegans: a complex cednario. *Nat. Rev. Mol. Cell Biol.*, 7(2):97–108.

Letunic, I., Copley, R. R., Pils, B., Pinkert, S., Schultz, J., and Bork, P. (2006). Smart 5: domains in the context of genomes and networks. *Nucleic Acids Res*, 34(Database issue):D257–60.

Li, J., Mahajan, A., and Tsai, M.-D. (2006). Ankyrin repeat: a unique motif mediating protein-protein interactions. *Biochemistry*, 45(51):15168–78.

Lin, F., Blake, D. L., Callebaut, I., Skerjanc, I. S., Holmer, L., McBurney, M. W., Paulin-Levasseur, M., and Worman, H. J. (2000). Man1, an inner nuclear membrane protein that shares the lem domain with lamina-associated polypeptide 2 and emerin. *J Biol Chem*, 275(7):4840–7.

Liu, J., Ben-Shahar, T. R., Riemer, D., Treinin, M., Spann, P., Weber, K., Fire, A., and Gruenbaum, Y. (2000). Essential roles for caenorhabditis elegans lamin gene in nuclear organization, cell cycle progression, and spatial organization of nuclear pore complexes. *Mol Biol Cell*, 11(11):3937–47.

Liu, J., Lee, K. K., Segura-Totten, M., Neufeld, E., Wilson, K. L., and Gruenbaum, Y. (2003). Man1 and emerin have overlapping function(s) essential for chromosome segregation and cell division in caenorhabditis elegans. *Proc Natl Acad Sci USA*, 100(8):4598–603.

Liu, Q. A. and Hengartner, M. O. (1999). The molecular mechanism of programmed cell death in c. elegans. *Ann N Y Acad Sci*, 887:92–104.

M

Margalit, A., Brachner, A., Gotzmann, J., Foisner, R., and Gruenbaum, Y. (2007a). Barrier-to-autointegration factor – a baffling little protein. *Trends in Cell Biology*, 17(4):202–208.

Margalit, A., Neufeld, E., Feinstein, N., Wilson, K. L., Podbilewicz, B., and Gruenbaum, Y. (2007b). Barrier to autointegration factor blocks premature cell fusion and maintains adult muscle integrity in c. elegans. *J Cell Biol*, 178(4):661–73.

Margalit, A., Segura-Totten, M., Gruenbaum, Y., and Wilson, K. L. (2005). Barrier-to-autointegration factor is required to segregate and enclose chromosomes within the nuclear envelope and assemble the nuclear lamina. *Proc Natl Acad Sci USA*, 102(9):3290–5.

Martin, J. S., Winkelmann, N., Petalcorin, M. I. R., McIlwraith, M. J., and Boulton, S. J. (2005). Rad-51-dependent and -independent roles of a caenorhabditis elegans brca2-related protein during dna double-strand break repair. *Mol Cell Biol*, 25(8):3127–39.

Meier, P., Finch, A., and Evan, G. (2000). Apoptosis in development. *Nature*, 407(6805):796–801.

Metzstein, M. M., Stanfield, G. M., and Horvitz, H. R. (1998). Genetics of programmed cell death in c. elegans: past, present and future. *Trends Genet*, 14(10):410–6.

Moolenaar, G. F., van Rossum-Fikkert, S., van Kesteren, M., and Goosen, N. (2002). Cho, a second endonuclease involved in escherichia coli nucleotide excision repair. *Proc Natl Acad Sci USA*, 99(3):1467–72.

Morinaga, H., Yonekura, S., Nakamura, N., Sugiyama, H., Yonei, S., and Zhang-Akiyama, Q. (2009). Purification and characterization of caenorhabditis elegans nth, a homolog of human endonuclease iii: Essential role of n-terminal region. *DNA Repair*.

N

Nyberg, K. A., Michelson, R. J., Putnam, C. W., and Weinert, T. A. (2002). Toward maintaining the genome: Dna damage and replication checkpoints. *Annu Rev Genet*, 36:617–56.

O

O'Neil, N. and Rose, A. (2006). Dna repair. *WormBook*, page 12.

Opferman, J. T. (2008). Apoptosis in the development of the immune system. *Cell Death Differ*, 15(2):234–42.

P

Park, H. K., Yook, J.-S., Koo, H.-S., Choi, I. S., and Ahn, B. (2002). The caenorhabditis elegans xpa homolog of human xpa. *Mol Cells*, 14(1):50–5.

Parrilla-Castellar, E. R., Arlander, S. J. H., and Karnitz, L. (2004). Dial 9-1-1 for dna damage: the rad9-hus1-rad1 (9-1-1) clamp complex. *DNA Repair*, 3(8-9):1009–14.

Pietsch, E. C., Sykes, S. M., Mcmahon, S. B., and Murphy, M. E. (2008). The p53 family and programmed cell death. *Oncogene*, 27(50):6507–21.

Praitis, V., Casey, E., Collar, D., and Austin, J. (2001). Creation of low-copy integrated transgenic lines in caenorhabditis elegans. *Genetics*, 157(3):1217–26.

R

Roldán-Arjona, T. and Sedgwick, B. (1996). Dna base damage induced by ionizing radiation recognized by escherichia coli uvrabc nuclease but not nth or fpg proteins. *Molecular Carcinogenesis*, 16(4):188–96.

S

Sancar, A., Lindsey-Boltz, L. A., Unsal-Kacmaz, K., and Linn, S. (2004). Molecular mechanisms of mammalian dna repair and the dna damage checkpoints. *Annu Rev Biochem*, 73(1):39–85.

Satoh, M. S., Jones, C. J., Wood, R. D., and Lindahl, T. (1993). Dna excision-repair defect of xeroderma pigmentosum prevents removal of a class of oxygen free radical-induced base lesions. *Proc Natl Acad Sci USA*, 90(13):6335–9.

Schertel, C. and Conradt, B. (2007). C. elegans orthologs of components of the rb tumor suppressor complex have distinct pro-apoptotic functions. *Development*, 134(20):3691–3701.

Schetter, A., Askjaer, P., Piano, F., Mattaj, I., and Kemphues, K. (2006). Nucleoporins npp-1, npp-3, npp-4, npp-11 and npp-13 are required for proper spindle orientation in c. elegans. *Dev Biol*, 289(2):360–71.

Schultz, J., Milpetz, F., Bork, P., and Ponting, C. P. (1998). Smart, a simple modular architecture research tool: identification of signaling domains. *Proc Natl Acad Sci USA*, 95(11):5857–64.

Schumacher, B., Hofmann, K., Boulton, S., and Gartner, A. (2001). The c. elegans homolog of the p53 tumor suppressor is required for dna damage-induced apoptosis. *Curr Biol*, 11(21):1722–7.

Schumacher, B., Schertel, C., Wittenburg, N., Tuck, S., Mitani, S., Gartner, A., Conradt, B., and Shaham, S. (2005). C. elegans ced-13 can promote apoptosis and is induced in response to dna damage. *Cell Death Differ*, 12(2):153–161.

Speliotes, E. K., Uren, A., Vaux, D., and Horvitz, H. R. (2000). The survivin-like c. elegans bir-1 protein acts with the aurora-like kinase air-2 to affect chromosomes and the spindle midzone. *Mol Cell*, 6(2):211–23.

Stergiou, L., Doukoumetzidis, K., Sendoel, A., and Hengartner, M. O. (2007). The nucleotide excision repair pathway is required for uv-c-induced apoptosis in caenorhabditis elegans. *Cell Death Differ*, 14(6):1129–38.

Stergiou, L. and Hengartner, M. O. (2004). Death and more: Dna damage response pathways in the nematode c. elegans. *Cell Death Differ*, 11(1):21–8.

Suh, E.-K., Yang, A., Kettenbach, A., Bamberger, C., Michaelis, A. H., Zhu, Z., Elvin, J. A., Bronson, R. T., Crum, C. P., and Mckeon, F. (2006). p63 protects the female germ line during meiotic arrest. *Nature*, 444(7119):624–8.

Sulston, J. E. and Horvitz, H. R. (1977). Post-embryonic cell lineages of the nematode, caenorhabditis elegans. *Dev Biol*, 56(1):110–56.

Sulston, J. E., Schierenberg, E., White, J. G., and Thomson, J. N. (1983). The embryonic cell lineage of the nematode caenorhabditis elegans. *Dev Biol*, 100(1):64–119.

T

Takanami, T., Mori, A., Takahashi, H., and Higashitani, A. (2000). Hyper-resistance of meiotic cells to radiation due to a strong expression of a single reca-like gene in caenorhabditis elegans. *Nucleic Acids Res*, 28(21):4232–6.

Thompson, H. M., Skop, A. R., Euteneuer, U., Meyer, B. J., and McNiven, M. A. (2002). The large gtpase dynamin associates with the spindle mid-zone and is required for cytokinesis. *Curr Biol*, 12(24):2111–7.

Tijsterman, M., Pothof, J., and Plasterk, R. H. A. (2002). Frequent germline mutations and somatic repeat instability in dna mismatch-repair-deficient caenorhabditis elegans. *Genetics*, 161(2):651–60.

Truglio, J. J., Rhau, B., Croteau, D. L., Wang, L., Skorvaga, M., Karakas, E., Dellavecchia, M. J., Wang, H., Houten, B. V., and Kisker, C. (2005). Structural insights into the first incision reaction during nucleotide excision repair. *EMBO J*, 24(5):885–94.

V

Verbrugghe, K. J. C. and White, J. G. (2004). Spd-1 is required for the formation of the spindle midzone but is not essential for the completion of cytokinesis in c. elegans embryos. *Curr Biol*, 14(19):1755–60.

W

Wagner, N. and Krohne, G. (2007). Lem-domain proteins: new insights into lamin-interacting proteins. *International Review of Cytology*, 261:1–46.

Whitehurst, A. W., Bodemann, B. O., Cardenas, J., Ferguson, D., Girard, L., Peyton, M., Minna, J. D., Michnoff, C., Hao, W., Roth, M. G., Xie, X.-J., and White, M. A. (2007). Synthetic lethal screen identification of chemosensitizer loci in cancer cells. *Nature*, 446(7137):815–9.

Wicks, S. R., Yeh, R. T., Gish, W. R., Waterston, R. H., and Plasterk, R. H. (2001). Rapid gene mapping in caenorhabditis elegans using a high density polymorphism map. *Nat. Genet.*, 28(2):160–4.

Z

Zheng, R., Ghirlando, R., Lee, M. S., Mizuuchi, K., Krause, M., and Craigie, R. (2000). Barrier-to-autointegration factor (baf) bridges dna in a discrete, higher-order nucleoprotein complex. *Proc Natl Acad Sci USA*, 97(16):8997–9002.

Zhou, B. B. and Elledge, S. J. (2000). The dna damage response: putting checkpoints in perspective. *Nature*, 408(6811):433–9.

Zipperlen, P., Nairz, K., Rimann, I., Basler, K., Hafen, E., Hengartner, M., and Hajnal, A. (2005). A universal method for automated gene mapping. *Genome Biol*, 6(2):R19.

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